Vol. 102, No. 2 April, 1952

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

STUDIES ON THE PIGMENTATION CHANGES DURING RECONSTITUTION IN TUBULARIA

ADOLPH I. COHEN

Marine Biological Laboratory, Woods Hole, Mass., and Department of Zoology, Columbia University, New York 27, N. Y.

A noteworthy feature during reconstitution of the marine hydrozoan Tubularia is the appearance of a reddening at the site of hydranth formation. This apparent concentration of pigment suggests a possible relation of the pigment to the metabolic processes of reconstitution.

Moreover, studies on the mechanism of dominance in this form have suggested to Barth (1938) that there is a competition by the ends of a stem piece for something in the circulation. When the circulation is blocked by oil drops (Barth, 1938), glass rods (Rose and Rose, 1941), or ligatures (Barth, 1938), dominance is obliterated. When an open capillary tube of short length is inserted into the coelenteron and the stem ligated about it, thus breaking the coenosarcal connection but temporarily keeping some fluid exchange, a dominance effect was noted by Rose and Rose (1941).

Since pigment granules are very evident in the circulation during regeneration, an investigation of the origin, fate and nature of the pigment was undertaken.

Material and Methods

The work reported here was done on Tubularia supplied by the Marine Biological Laboratory at Woods Hole during August, 1951. The stems were four to eight inches long and unbranched. The hydranths show the tentacle characteristics reported for *Tubularia crocea*. The coelenterons were usually partitioned, starting just below the hydranth constriction, by tissue sheets connecting the gastric ridges. These sheets may connect adjacent ridges, in which case the cross-section shows cords less than a diameter, or several sheets may seem to originate from a center giving a wheel-hub effect in cross-section (Fig. 1, C and D). The flagellated cells responsible for fluid circulation are mainly at the two junctions of any gastric ridge with the rest of the coenosarc as seen in cross-section.

The pigment of Tubularia is typically red. It has been long observed that this redness is variable but an unusual event, noticed by the Woods Hole Supply Department and by us for the first time in August, 1951, is that interspersed with the red stems were yellow and orange stems, as well as intergrades. These stems were in no apparent way otherwise different from the red ones. The colors occurred in both young and old hydranths, both sexes, and in stems of differing lengths. It

was not unusual to find a yellow stem in the midst of red stems. These individuals offered a new approach to the problem of pigmentation during regeneration.

Unless otherwise noted, all stem fragments were 5 mm. in length and were taken from a point 3 mm. behind the hydranth constriction. To keep track of the original polarity, the distal cut is made horizontal and the proximal oblique. Where a group of four or less stems was being followed, the stems were placed in 50-ml. Erlenmeyer flasks half filled with sand-filtered sea water. Where 5 to 10 stems were

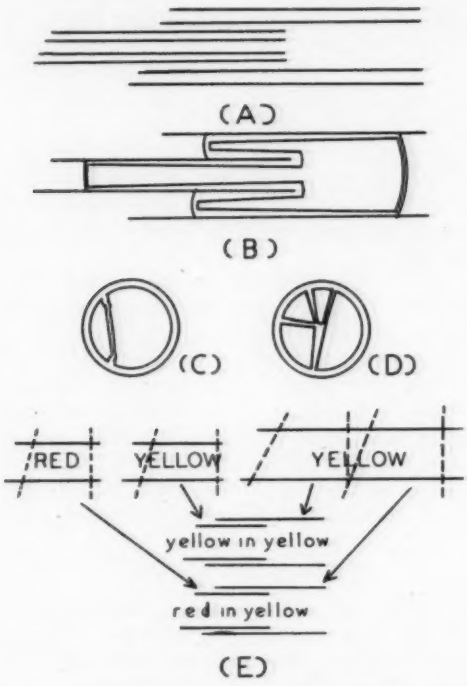


FIGURE 1. For explanation, see text.

being followed as a group, they were kept in fingerbowls in 100 ml. of filtered sea water. All containers were placed in an illuminated cold room at 16–18° C. The average time for regeneration of a hydranth was about three days.

EXPERIMENTAL

Preliminary experiments involving regeneration of sections from hydranths of colors other than red showed that without exception the regenerant was of the same color as the original. In some cases a decrease in color intensity seems to have occurred, but this is not clear cut. An experiment was then performed

wherein portions of red stems were allowed to fuse with yellow stem fragments, to determine the influence of this situation on the color of the hydranth forming in the yellow component.

All yellow stems were taken from animals bearing yellow hydranths, and all red stems from animals bearing red hydranths. Hydranth and stem color always corresponded, although the intensity was both less and somewhat more variable in the stem portion.

For the fusion experiment, stems were selected which were either intensely red or pale yellow. For each set in this experiment, one yellow and one red stem of approximately equal diameter, and a second yellow stem of larger diameter were used (Fig. 1, E). Two 3 mm. fragments were cut from the larger stem and one each from the smaller stems. Under the binocular microscope, the distal end of the red piece was inserted into the proximal end of one of the large yellow pieces, and similarly the distal end of the small yellow fragment was inserted into the proximal end of the other large yellow piece. The original polarity was therefore maintained. Every effort was made to align the gastric ridges of the pieces, since preliminary experiments showed that failure to do so tended to prevent fusion of the fragments. It was considered preferable to insert the red stem into the yellow one rather than the converse, since it is possible that the hard perisarc of the insert might traumatize the coenosarc of the containing piece and liberate pigment. A few such converse experiments will be reported as controls. The use of the large yellow fragment as the containing piece also had the benefit of tending to make the hydranths form in the yellow component, since the fused 3 mm. fragments behave like a single 6 mm. stem piece in exhibiting dominance. The healing process is shown diagrammatically in Figure 1, A and B. One might largely insure formation of the hydranth in the yellow end by ligation of the red end, but ligation definitely damages tissue and so this was avoided. The colors of the regenerated hydranths were examined against a black background with strong white side lighting. The colors are unambiguous. The results are presented in Table I.

The significant results are those in which (1) successful fusion has occurred, with the coelenteron being common to both components, and (2) the formation of the hydranth has occurred in the yellow end of a red stem fused to a yellow stem. It will be noted that in every such case the hydranth was redder than the hydranth of the control consisting of two fused yellow pieces.

Observations of fused stems during the regeneration process show that the red pigment appears in the circulation following healing of the cut ends. It then seemingly is picked up by the gastric ridges. The gastric ridges of the yellow components can be seen with lines of red particles. This effect varies in uniformity since the gastric ridges do not occur symmetrically and the circulation currents vary in rate of flow and may have eddies. As noted by Stevens (1901) there is a tendency for the gastric ridges to break down as regeneration proceeds and the picked-up pigment tends to be re-liberated. When the primordia of the tentacles form, however, it can be inferred that pigment particles are picked up by them, as chains of these particles mark the tentacular ridges. There are also some randomly distributed particles in this area.

The above experiment was repeated in a few cases with oil drops placed in the coelenteron of the yellow components before healing occurred. These drops block

the circulation. In these cases the hydranth color was the same as that of the control hydranth. This might also be held to rule out a factor transmitted by the red component to the yellow via the coenosarc. The oil drop, however, may affect the coenosarc adjacent to it by limiting the entering and leaving of metabolically important materials, and thus effectively also block the coenosarc pathway.

TABLE I

A: Success of Fusions

All fusions successful and normal except:

Set No.	Yellow to Yellow	Yellow to Red
7	S	U
11	U, cytolyzed	U, abnormal
12	U	S
15	S, abnormal	S
26	U	S, abnormal

B: Observed Color of Hydranths

Fusion: Hydranth-bearing end: Set No.	Yellow in Yellow	Red in Yellow		Yellow in Red	
	Either	Red	Yellow	Yellow	Red
1	Y	O	O		
2	Y	R			
3	O		OR		
4	O				OR
5	Y		O		
6	Y			Y	
7	O	R	Y		
8	YO	OR			
9	Y		O		
10	YO			OR	
11	—	O			
12	Y			OR	OR
13	Y	R			
14	YO		OR		
15	Y		O		
16	Y		O		
17	Y		OR		
18	Y		OR		
19	Y		O		
20	Y	OR			
21	Y		O		
22	YO		OR		
23	Y	OR			
24	Y	O	O		
25	Y	OR	O		
26	Y	OR			

KEY: S—successful; U—unsuccessful; Y—yellow; YO—yellow-orange; O—orange; OR—orange-red; R—Red.

Since one could not see individual particles being picked up, the possibility also remained that some soluble pigment precursor was being produced by the red stem and entering the yellow. Attempts to insert filters of various sorts into the coelenteron failed and the procedure was intrinsically undesirable, as it was impossible to place these without severe damage to the gastric ridges and partitions.

A probable resolution of these questions was effected in the following manner. "Norite" animal charcoal was ground in sea water with a mortar and pestle and a suspension of fine particles injected with a micropipette into the coelenteron of various stem pieces. These particles tend to adhere to the gastric ridges. Following healing, the particles become loose with breakdown of the ridge and are readily seen in circulation. This might simply be a washing free as circulation begins, but the disappearance of spot stains of Nile blue sulphate suggested an actual breakdown. With regeneration the charcoal particles tend to adhere in the area of hydranth formation and particle chains delineate the primordia of the tentacles. In one case where a stolon started to form, a tendency was noted for the charcoal particles to adhere at this area. In every respect the distribution of red pigment particles and charcoal particles is the same.

Smears of emerged hydranths following charcoal treatment show that the pigment masses and charcoal are similarly localized in the endoderm and between the endoderm and ectoderm layers. Sections of forming and emerged hydranths, following Zenker's fixation and staining with carefully filtered Heidenhain's haematoxylin without counterstain, confirm the charcoal distribution as observed in smears. Small charcoal fragments appear to be inside some endoderm cells. No mitoses were evident in the regenerating areas.

The above observations are regarded as making it highly probable that pigment particles are actually picked up. It was then decided to examine the character of the circulating fluid.

Stevens (1901) reports the contents of the circulation as pigment granules, translucent globules and nuclei. Hargitt (1903) records the presence in the same species of cnidocysts in the endoderm and circulation. We have examined the character of the fluid withdrawn from circulation with a fine pipette and confirm the presence of cnidocysts, pigment granules and cell debris. The material in circulation varies according to the time after healing when the fluid is examined. This may account for the failure of Stevens to find cnidocysts.

To determine the origin of the circulating material, smears of stem tissue were examined for obviously specialized cell types. In the endoderm were seen numerous cnidocyst cells and round or oval pigment cells. The latter are generally yellow but frequently have in them darker orange or red masses suggesting storage or concentration of this material.

In the course of regeneration, the area at an end where a hydranth is not forming becomes transparent; it was therefore decided to check to see whether this was an actual thinning or simply a loss of opaque material such as pigment. An actual thinning might be due to local cytolysis or a contraction of tissue into other areas, or to cell migrations. Therefore, sections of stems were cut and immediately thereafter additional cylinders approximately 1 mm. in length were cut from the remaining stem just proximal and just distal to the 5 mm. fragment. Working rapidly, these were immediately stood on end and the tissue thickness measured, using an ocular micrometer. The magnification was 43 × and 1 mm. of tissue was 10 micrometer units. This procedure is not adequate for small changes; however, it was readily observed that changes of the magnitude of a 50% decrease in coenosarc thickness occurred when, during regeneration, 1 mm. cylinders were cut from the stem piece up to the area of hydranth formation. It was not possible to judge with

certainty whether a gradient of thickness, showing increase in the direction of the hydranth, occurred although this is the visual impression. The need for speed in measurement is due to the fact that with the onset of the healing process the cut ends curl inward and increase in optical cross-section. The hydranth-forming area, as expected, becomes very thick and almost obliterates the coelenteron. Measurements were made on perisarc thickness concurrently as a check on technique, and these showed no change.

Chemical studies of a preliminary nature were then made on red stems, only, in an effort to obtain some information about the pigment. The pigment proved to be water insoluble but soluble in organic solvents, particularly polar solvents such as acetone, methanol and pyridine. With concentrated sulfuric acid a blue color is obtained. A chloroform solution gives a positive Carr-Price test for carotenoid by giving a blue-green color. Absorption measurements were made on various extracts using a Beckman spectrophotometer. In every case, single peaks in the visible range were obtained. These were: 490–500 mμ in pyridine; 455–70 mμ in petrol ether; and 470–80 mμ in acetone. The only carotenoid listed in Karrer and Jucker (1950) showing such characteristics is astacene. Astacene's solubility properties are very similar to those of the red pigment. Astacene is a pigment occurring mainly in animals, only one case being known of its presence in a plant, an alga. It is, however, supposedly an extraction artefact of astaxanthin and results from oxidation during extraction. Attempts to obtain astaxanthin by homogenizing hydranths in acetone-solid CO_2 slush and extracting under nitrogen failed but are not regarded as being final.

The above extracts were made on hydranths only, since numerous red algae and other organisms grow on the perisarc. It was noted, however, during the course of tissue smears, that our material was frequently infected with red algae in the endoderm. The most common of these resembles *Ceramium*.

As a preliminary to future work, an extract of hydranths of all colors in petrol ether was chromatogrammed on a sucrose column. Three red bands, one yellow band and one orange band were noted, but no attempt was made to collect the fractions. The bands may be due to different chemical chromatophores, to different esterified combinations of the same chromatophore, or to natural or extraction-created isomers.

Discussion

Loeb (1891), who first noted the color changes in regeneration, suggested that the pigment might be nutritive. Driesch (1900), noting that the pigment particles seemed to be linearly arranged following the tentacular primordia, believed that they might have morphogenetic significance. Morgan (1901) challenged this, however, on the basis that despite evident considerable variation in pigmentation, regeneration proceeded normally. He also noted that the stolon frequently showed the pigment.

There is still no evidence of any functional significance for the pigment of Tubularia. The non-random arrangentent of pigment particles seen by Driesch as delineating the tentacular primordia is now seen to be due to a mechanism which indiscriminately takes up particles. The presence of cnidocysts in the endoderm and in circulation suggests that this may be the normal method of supplying the

cnidocyst units of the new hydranth. The presence of charcoal particles between the two layers but not in the ectoderm would then suggest that the mechanism is particle-indiscriminate up to this point, but that incorporation into the ectoderm involves discrimination.

Against this concept it must be noted that charcoal and pigment tend to adhere where the stolon begins to form. This may mean that these growing areas simply have sticky surfaces and that the lining-up of particles in the tentacular primordia is the incidental result of cell movements.

Stevens (1901) and Godlewski (1904) believe that the red pigment is liberated by endoderm cytolysis, particularly of the gastric ridges, during regeneration, and is a waste material expelled when regeneration is complete. We confirm the liberation of some pigment with gastric ridge breakdown.

The pigment may indeed be a waste product derived from ingested crustaceans who, in all probability, contain astacene (or astaxanthin). There is no denial that some is frequently expelled after reconstitution. Preliminary chromatography does suggest, however, that it may pass through several chemical stages in Tubularia, as is also suggested by the occurrence of strikingly different colored specimens in the same clump of stems. These could be biochemical mutants.

The storage of pigment in Tubularia might serve as a prey-attracting mechanism. No evidence exists on this point. Carotenoid pigments are of known importance in visual biochemistry. There is no evidence on light sensitivity in this form. Carotenoid materials have been reported as gamete-attracting agents (Moewus, 1940). While this has not been confirmed, the pigment here occurs in both sexes and in apparently similar distribution.

Regarding the chemical nature of the pigment, Driesch (1900) remarks that it was identified for him as a carotenoid, and Lönnberg and Hellström (1931), working with *T. larynx,* name it as astacene. However, they report a main absorption band in petrol ether at 432–4 mμ and minor bands at 458–9 mμ and 488–91 mμ. Our work confirms their identification but not the presence of three bands. Our data are in agreement with the spectral and other characteristics described by Karrer and Jucker (1950) for astacene.

It is undisputed that the area of hydranth formation shows a prior reddening, and the latter must be due to one or more of the following events: 1) local synthesis of pigment; 2) local acquisition of pigment formed elsewhere; and 3) more pigmented cells entering the area. Therefore, histological studies of the cell movements, divisions and growth relative to regeneration are important. Histological studies have been made by Bickford (1894), Stevens (1901), Godlewski (1904) and Hargitt (1903). Alone among these, Stevens reports mitotic figures in the regenerating area, both in ectoderm and endoderm. She also noted that in her sections the tissue in the non-regenerating area had become thin and the cells elongated in the stem length. To her, this suggested contraction of cells into the regenerating area. Contraction would account for reddening by increasing the length of the optical path through the pigmented material. This is readily duplicated by ramming tissue in the perisarc with a glass rod. Hargitt, unlike Stevens, finds no mitoses and believes that amitotic divisions must occur. Cell divisions are necessary to account for the increase in ectoderm surface, according to Hargitt. Bickford's work is directed towards the successful establishment of the view that the

hydranth must form from previously differentiated cells, rather than from a primitive cell, but she does not touch the problem of which differentiated cells form the hydranth. Against the contraction hypothesis of Stevens is the old observation of Allmann (1874) that no muscle cells exist in the tissue under the perisarc. This is not necessarily a valid objection since all cells are contractile to some degree.

Our work confirms the decrease in tissue thickness and the absence of distinguishable mitotic figures which therefore would lend weight to a cell movement theory. It must be pointed out, however, with regard to the observation regarding mitoses, that it is possible that we are dealing with mitoses of an unusual type. Mitoses might occur in waves, for example, and thus not be picked up in sections made on 5 or 6 stems. This would explain Stevens' finding them and the failure of the other observers to do so. Work with agents which arrest mitoses might clarify this point.

One still may not say that the reddening which marks the site of hydranth formation is entirely due to picked-up pigment. While this and intrinsic pigment could account for a good part of the reddening, contraction of tissue into the area is not ruled out. The discreteness of the particles marking the tentacular primordia, however, suggests that the former originate entirely from picked-up pigment.

Summary

1. At least part of the reddening designating the site of hydranth formation in Tubularia is due to pigment removed from circulation.

2. The mechanism removing pigment particles from circulation does not distinguish them from charcoal particles introduced into the stem. These become distributed in exact coincidence with the pigment in the reconstituted hydranth.

3. The extracted pigment of Tubularia seems to be mainly astacene, as judged from its spectral and solubility properties.

4. The immediate origin of the pigment in circulation is from the breakdown of endodermal tissue.

5. The possible significance of removal of pigment is discussed.

6. Color variants in Tubularia are noted, as well as infecting red algae.

LITERATURE CITED

Allmann, G. J., 1874. A monograph on the Tubularian hydroids. Pt. I. Published for the Ray Society by R. Hardwick, London.

Barth, L. G., 1938. Quantitative studies of the factors governing the rate of regeneration in Tubularia. *Biol. Bull.*, **74**: 155–177.

Bickford, E., 1894. Notes on regeneration and heteromorphosis of Tubularian hydroids. *J. Morph.*, **9**: 417–430.

Driesch, H., 1900. Studien über das Regulationsvermögen der Organismen. 2. Quantitative Regulationen bei der Reparation der Tubularia. *Arch. f. Entw.*, **9**: 103–136.

Godlewski, E., 1904. Zur Kenntnis der Regulationsvorgänge bei Tubularia mesembryanthemum. *Arch. f. Entw.*, **18**: 111–160.

Hargitt, G. T., 1903. Regeneration in Hydromedusae. *Arch. f. Entw.*, **17**: 64–91.

Karrer, P., and E. Jucker, 1950. Carotenoids. Elsevier, New York.

Loeb, J., 1891. Untersuchungen zur physiologischen Morphologie der Thiere. I. Über Heteromorphosis. Würzberg. Heading 6, pp. 29–30.

Lönnberg, E., and H. Hellström, 1931. Zur Kenntnis der Carotinoids bei marinen Evertebraten. *Ark. Zool.*, **23**: 1–74.

MOEWUS, F., 1940. Carotinoid-Derivate als geschlechtsbestimmende Stoffe von Algen. *Biol. Zentralbl.*, **60**: 143–166.

MORGAN, T. H., 1901. Regeneration in Tubularia. *Arch. f. Entw.*, **11**: 346–381.

ROSE, S. M., AND F. C. ROSE, 1941. The role of a cut surface in Tubularia regeneration. *Physiol. Zool.*, **14**: 323–343.

STEVENS, N., 1901. Regeneration in Tubularia mesembryanthemum. I. *Arch. f. Entw.*, **13**: 410–415.

STEVENS, N., 1902. Regeneration in Tubularia mesembryanthemum. II. *Arch. f. Entw.*, **15**: 319–326.

THE BIOLOGY OF THE SIERRA LUMINOUS MILLIPEDE, LUMINODESMUS SEQUOIAE, LOOMIS AND DAVENPORT

DEMOREST DAVENPORT, DONALD M. WOOTTON AND JOHN E. CUSHING[1]

Dept. of Biological Sciences, University of California, Santa Barbara College, Santa Barbara, California

In May of 1949 a group of students on an ecology field trip to the Sequoia National Forest (Camp Nelson, Tulare Co., California) brought back to the senior author a large number of striking and brilliantly luminescent millipedes. These animals were sent to Dr. E. Newton Harvey of Princeton University, who in turn forwarded them for identification to Mr. H. F. Loomis of the U. S. Plant Introduction Garden, Coconut Grove, Florida. Mr. Loomis found them to constitute an undescribed genus and species, and there resulted a collaborative original description (Loomis and Davenport, 1951). Reports of luminous millipedes are few. Bruner (1891) observed and described animals in Nebraska which were later given the name *Fontaria luminosa* by Kenyon (1893) and thus placed in the family Xystodesmidae, in which Luminodesmus belongs. It would appear, however, that these studies constitute the first investigations to be made of the life-history and physiology of luminescence of a luminous diplopod.

Luminodesmus sequoiae is a large and handsome millipede, the adults averaging 40 mm. in length and 8 mm. in width, of a pale tan or salmon color in late adult life, with a dark mid-dorsal line. In darkness the entire animal and its appendages give off a brilliant greenish-white glow, plainly visible to the dark-adapted eye at a considerable distance. Figure 1A is a photograph in ordinary (stroboscopic) illumination, while Figure 1B shows the animal taken in its own light only.

After discovery and description of the millipede, it became apparent that a thorough investigation of its ecology, distribution, life-history and physiology should be undertaken. Accordingly, in May of 1951 a trip was made to the type-locality for the purpose of collecting numbers of living animals, and some four hundred or more were brought back to Santa Barbara.

In the laboratory the millipedes proved easy to culture. A minimum of care was necessary to keep them alive and to raise the early stages. Adults were kept in a series of standard glass terraria filled to a depth of 5 or 6 inches with the rich, dark humus collected in their habitat. These terraria were covered with glass sheets and sealed with vaseline; about every 10 days they were opened and the humus

[1] The authors wish to express their appreciation to Dr. E. Newton Harvey of Princeton University for suggestions and advice; to Dr. William D. McElroy of Johns Hopkins University for providing the firefly preparations; to Messrs. Theodore Gayas and Richard Addison of the Brooks Institute of Photography, Santa Barbara, for the photographs; and to Campbell Grant of Carpinteria, California for his execution of the accompanying drawings. The technical assistance of Eugene Sheller is also gratefully acknowledged.

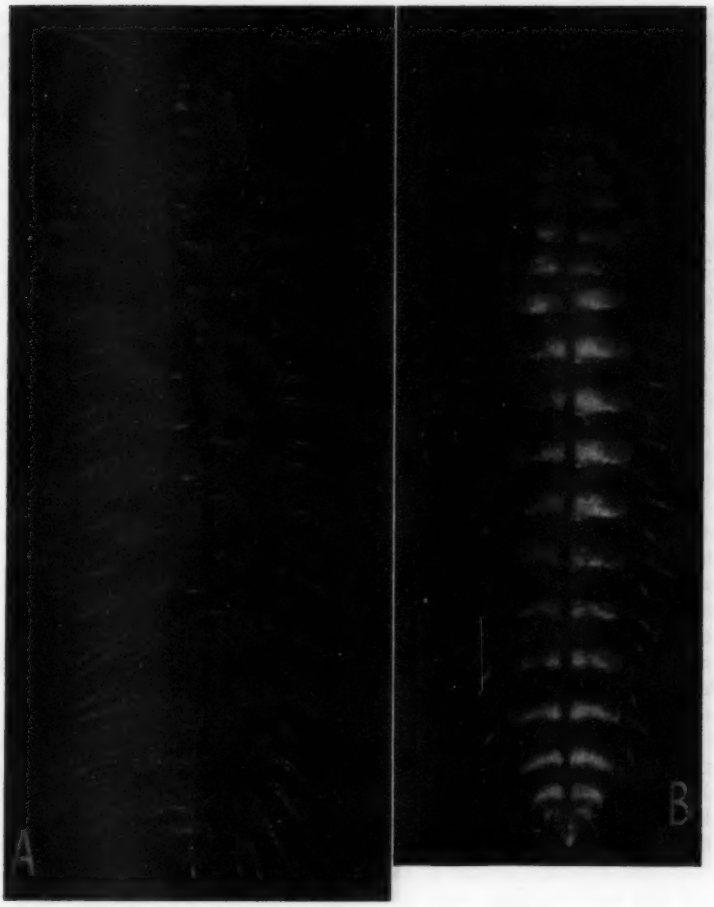


FIGURE 1. A. Adult *Luminodesmus sequoiae*. Approx. × 3. B. An adult *L. sequoiae* photographed in its own light. Animal curarized. Approx. × 3.

lightly sprinkled with water. Occasionally, when animals were removed for experimental purposes or a search for early stages was made, fresh humus was added from storage containers brought in from the field at the time the animals were collected.

ECOLOGY AND DISTRIBUTION

Luminodesmus sequoiae appears to have a very limited geographic range. So far the only records are one from the type-locality and an uncertain one from

Kaweah, Tulare Co. (Loomis and Davenport, 1951). During the summer of 1951 a brief visit was made to Yosemite, Kings Canyon and Sequoia National Parks during which it was found that no such animals had ever been observed in the parks to the knowledge of the Park Naturalists and their staffs. One ranger stated that he had observed "large luminescent animals" on a trail along the Merced River at low altitudes in Yosemite, but that these were not identified,

It appears probable that the animals may be limited to a particular environment with the characteristics of the type-locality, relatively moist with a thick, dark humus under a leafy litter made up of the disintegrating leaves of *Quercus, Acer,* etc., with an admixture of conifer needles. They must occur rarely if at all in the heavy coniferous forests of higher altitudes in the Sierra, characteristic of Kings Canyon and Sequoia National Parks; otherwise it would seem that they could scarcely have escaped notice. However, it is likely that further investigation of areas similar ecologically to the type-locality at lower altitudes within the Parks and in the foothills beyond their western limits may extend the range of Luminodesmus.

As has been stated, at the type-locality the animals may be so numerous on the surface that at night, when their bright patch of light attracts the eye, dozens can soon be collected. An effort was made in May of 1951 by Dr. E. R. Noble of the collecting group to determine how common the developmental stages might be in the upper layers of humus and soil. It was found that their distribution was extremely irregular. Several areas two feet square were carefully sorted over to a depth of 6 inches. In one such area 54 animals, ranging in length from 2–35 mm., were collected. Some areas of similar size had a far smaller population and in others no millipedes whatever could be found. No very striking differences in the characteristics of these areas could be observed which might account for the difference in distribution. It is apparent that the animals may be numerous enough locally so that their feeding activities could have a marked effect on the characteristics of the humus in their particular niche.

An investigation at the type-locality in October of 1951 showed that at this late season after a summer of extreme dryness adults were almost totally absent. A careful search on two consecutive nights revealed no adults at the surface. During daylight a number of areas where large numbers had been taken the previous spring were carefully investigated by digging to a depth of approximately 9 inches. It was found that although certain of these areas, particularly under the overhanging edges of logs and boulders, were filled with well defined workings and broken cocoons, no adults of the preceding summer were present. In some areas, however, numbers of the last four larval stages were discovered, seemingly distributed at random in the first 6 inches of soil, while a number of recently moulted adults were also present. All living animals were very inactive and there were indications of a high mortality due to desiccation, since a number of intact cocoons containing the dried remains of animals were found.

It would appear, therefore, that very few adult animals survive the long period of desiccation from mid-summer until the beginning of the winter rains, at least in years of light rainfall. This observation is borne out by the comment of one of the district foresters, stationed at Camp Nelson, who on being questioned, said that he was familiar with the animal and that it was a creature of the spring rains, disappearing at the beginning of the summer drought.

Life-History

Millipedes that were kept in humus in the sealed terraria in the laboratory thrived for many months, burrowing through the humus in their feeding and coming actively to the surface at night to wander about. The room in which they were kept varied in temperature in the usual way during the day from approximately 65° F. to 85° F. Because of the relatively constant temperature of this artificial environment and the knowledge that at least during several months of their development in nature they are subject to daily extremes of temperature from well below freezing to above 80° F., it did not seem worthwhile to go to the difficulty of determining in more than a general way the time-duration of each stage.

When the adult animals were first brought into the laboratory in May they were placed in fresh humus known to be free of eggs or early stages. Although mating was not observed in the laboratory, it is reasonable to assume that it freely occurred, since members of both sexes were placed in the terraria at random. The terraria were first investigated for the presence of early stages on July 19th and numbers of egg-masses were found (Fig. 2A). The date of laying was not known. These egg-masses varied widely in the number of contained eggs; three that were counted contained approximately 160, 70 and 165 respectively. The masses were laid in

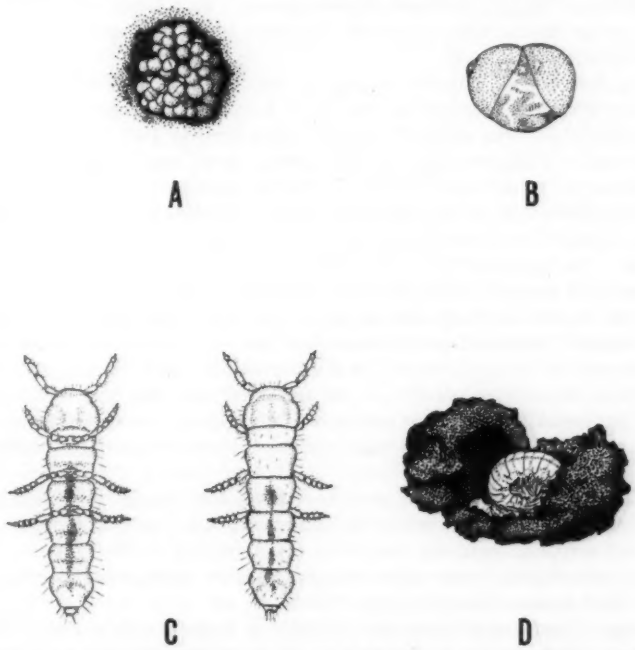


Figure 2. A. Egg-mass. Approx. × 2½. B. "Split-stage" egg. Approx. × 17. C. First instar larva in ventral and dorsal view. Approx. × 20. D. Opened cocoon of moulting larva. Approx. × 1¼.

loose cells of humus or in the closed ends of tunnel branches, a few inches below the surface. No firmly cemented cocoons of the type manufactured by moulting larvae appear to be constructed for these nests.

The eggs were approximately round, averaged 0.7 mm. in diameter, and appeared pearly, with a smooth surface. They were coated with a sticky substance that caused them to clump together, as has been described in other millipedes (Miley, 1927; Causey, 1943). As the eggs advanced in age the outer envelope took on a light brownish tinge.

In a number of egg clusters investigated, development had proceeded to a point which we have called the "split-stage" (Fig. 2B). In this stage, in which two halves of the split shell have been sprung apart like hinged hemispheres, there is contained a sac-like structure through which the developing larva with its appendages, etc., can just be perceived. Such a stage has been described for the juloid millipede, Arctobolus (Loomis, 1933) but not for the more closely related Polydesmid, Orthomorpha (Causey, 1943).

Loomis relates that in Arctobolus the first stage after hatching consists of a grub-like creature without appendages contained in a delicate semi-transparent membrane. We believe, although it could not be demonstrated, that in Luminodesmus this stage is passed briefly while in the "split-stage." When true hatching occurs, typical six-legged first instar larvae appear. In a large nest of "split-stage" eggs, first instar larvae were observed hatching from the membranous structure within the halves of the shell.

That the duration of time in the egg is not less than two weeks was demonstrated, since first instar larvae hatched from July 31st to August 3rd from "split-stage" eggs that had been isolated in small glass jars on July 19th.

No luminosity whatever could be observed in large egg-masses either before or in the "split-stage." All observations on luminescence were made in a dark-room after 15–20 minutes of dark-adaptation by means of Polaroid Corp. type-XDA8FAP red lenses. Single first instar larvae were checked for luminescence and none could be observed. An opportunity to check larvae hatching in numbers from a clump of eggs showed that enough luminescence is present in first instar larvae for it to be just apparent to the scanning, dark-adapted eye when the larvae are crowded together in a mass. Under these circumstances there is just enough luminosity present to be picked up with rod-vision in the thoroughly dark-adapted eye.

Whether luminosity is present in the egg itself has not been definitely determined. It is certainly not evident even when the eggs are in large masses; it is possible, however, that the opacity of the envelope prevents passage of light to the exterior.

The first instar larva may be seen in ventral and dorsal view in Figure 2C. This stage has 7 post-cephalic segments and averages 1.7 mm. in length. The animals are very active on hatching and move about feeding on the humus. At hatching they are transparently pale white, but shortly after commencing feeding, the gut, filled with black humus, becomes very evident.

A number of first instar larvae were isolated in humus on July 19th. By August 10th numbers of these were observed to have constructed the typical cemented cocoons in which moulting of larvae occurs. A cocoon broken open with a late larva *in situ* is shown in Figure 2D. The cocoon closely resembled that described

by Miley (1927) in Euryurus. Cocoons are ball-like, smooth on the inside and rough on the exterior, of a dark, smoothly homogenous consistency apparently the result of the working together of humus and earth with some secretion. These structures are entirely closed and some possess the chimney-like extensions described by Miley. It is not unusual to find more than one individual in a single cocoon, particularly in some of the early moulting stages. As other authors have described, the millipedes are extremely inactive when in the process of moulting in the cocoons. That there is a general slowing down in metabolism at this time is indicated by the marked decrease in luminosity. No animals have been observed to lose their luminosity at any time from larva to adult, but during moulting the intensity is greatly diminished.

On August 20th the same isolation jar was examined, and a number of second instar larvae had appeared and were actively feeding. The time duration in the first instar was therefore approximately 33 days. Second instar larvae had 6 pairs

TABLE I

Characteristics of developmental stages

	Pairs of legs		No. post-cephalic segments	Average length in mm.
	♂	♀		
1st stage	3	3	7	1.7
2nd stage	6	6	9	2.9
3rd stage	11	11	12	3.8
4th stage	16	17	15	6.5
5th stage	22	23	17	8.0
6th stage	26	27	18	13.4
7th stage	28	29	19	22.1
Adult	30	31	20	41.0

of appendages, 9 post-cephalic segments and averaged 2.9 mm. in length. Total luminosity of these animals had increased so much that the position of three animals in a Petri dish could clearly be observed after 10 minutes dark-adaptation.

On September 9th these second stage larvae were observed to have constructed cocoons and on September 20th a number of third stage larvae had appeared. These possessed 11 pairs of legs, 12 post-cephalic segments and averaged 3.8 mm. in length. The time duration of the second instar was approximately 31 days.

Subsequent moults appear to proceed in much the same way, although as this is written laboratory-raised larvae have only reached the fourth larval stage. Fifth, sixth and seventh stage larvae were collected in the field in May and October of 1951.

The characteristics of the developmental stages are summarized in Table I.

Gonopod development in the males of *Luminodesmus sequoiae* resembles that described for other polydesmoid millipedes. In the third larval stage no sexual dimorphism can be observed in the appendages. In the fourth larval stage, in place of the anterior pair of legs of the 7th post-cephalic segment there appear two very small raised domes (Fig. 3A). In the fifth larval stage these have become

slightly larger. In the sixth and seventh larval stages proximal and distal segments are plainly apparent, the former segment being sunk into a pit in the sternite (Fig. 3B). The fully developed gonopod appears in the adult (Fig. 3C).

FIGURE 3. A. Appendages of the 7th post-cephalic segment in male fourth larval stage, showing gonopods appearing in position of eighth leg-pair. B. Developing gonopods of the seventh instar. C. Gonopods of adult male. All approx. × 13.

THE SOURCE AND NATURE OF LUMINESCENCE

While an extensive study of the luminescence of *Luminodesmus sequoiae* has yet to be made, some observations of general interest can be presented at this time.

The luminescence is continuous and appears to be under no voluntary control. During periods of general inactivity (*e.g.*, moulting) a decrease in intensity may occur. Momentary variations in light intensity in the entire animal may occur, but at the present time it cannot be said with certainty that these changes are correlated with changes in bodily activity. It has often been noted that luminosity may fade within segments or groups of segments under abnormal conditions (such as preceding death from local damage, poisons, CO_2).

Several observers have stated that they had an impression of a particulate light source when they examined the brilliant dorsal integument. Figure 4, an enlargement of five segments taken in their own light, strengthens the impression that there are in the integument scattered points of more intense luminosity. It is possible, however, that this effect may result from the breaking up of uniform light coming from deeper layers by the surface sculpture of the integument. This photograph, as well as Figure 1B, was taken in complete darkness with a Leica f-2 on Super-XX panchromatic film. The exposure time was one hour. Animals were inactivated by injection of 0.25 cc. *d*-tubocurarine chloride at a concentration of 3 mg./cc. The neuro-muscular blocking agent appeared to have no direct effect on luminescence.

Efforts to determine the exact source of the luminescence have been made as follows. Dissection and observations have determined that the fat bodies, viscera, tracheae, musculature and blood are not luminous. Frozen sections of adult animals have been made with CO_2. If these are rapidly thawed out under a jet of oxygen and observed in darkness, it can be seen that the source of the luminescence lies in the integument, since a cross-sectional "pipe" of light can be observed. The

light from such sections fades out in a few minutes and is so pale and so diffuse as to make it impossible to resolve the source under the microscope. If the sclerotized integument of the animal is scraped with a scalpel on the outside so that flakes are removed, no trace of luminosity can be observed in these flakes. Further evidence that the source of the luminescence may lie in the deeper integumentary layers, perhaps the epidermis or endocuticle, is presented by the fact that the antennae and legs, containing non-luminous blood, muscle, etc., are brilliantly luminescent. Under the dissecting microscope in darkness, appendages appear to give a relatively uniform glow with the intensity appearing greater to the eye at the curving surfaces at the margins, where there is, of course, greater integumentary thickness.

Paraffin sections of adult animals and fourth stage larvae have been cut and stained with Mallory's triple stain with Orange G. It has so far been impossible to discern either in the endocuticle, which is traversed by numerous canals, or the epidermis, the granules which have so often been described in animal cells that are

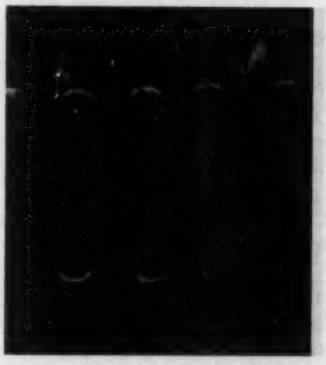


FIGURE 4. A dorsal view taken in animal's own light showing particulate appearance of light source and brilliance of lateral margins. Animal curarized. Approx. × 4.

auto-luminescent. Fresh, unstained smears of integument or of appendages, or smears stained with methylene blue have given no indication under oil-immersion of the presence of such bodies or of bacteria which might be suspected of being the source of luminescence.

Efforts to make extracts of Luminodesmus which would retain luminescence *in vitro* have so far been without success. Extracts prepared by grinding whole or eviscerated animals in water and filtering the extract into an evaporating dish glowed very briefly and went out. To date it has not been possible to re-activate the extract by adding various combinations of Photinus extract (including concentrated "luciferase" extracts made from freshly dried lanterns), magnesium ion and adenosine triphosphate (*cf.* McElroy and Harvey, 1951, for further references). Attempts to show classical "luciferin-luciferase" activity by combining heat-treated fractions with non-heat-treated ones (Harvey, 1940) have not been successful. However, it should be possible to study the effects of some factors on the emission of light in Luminodesmus in a semi-isolated system, since it has recently been found

that single legs and antennae if placed in distilled water will retain full luminosity for at least four hours and overnight if refrigerated.

As has been stated (Loomis and Davenport, 1951), if the animal is irradiated with an ultra-violet light source, a brilliant chartreuse-green fluorescence appears. This fluorescence comes from the entire integument and often most brilliantly from areas which are not as intensely luminescent as others, such as the ventral surface. Blood, viscera and trachae are not fluorescent. That a yellow-green fluorescent compound is directly involved in the luminescence of fire-flies has been demonstrated (Strehler and McElroy, 1949). However, it should be borne in mind that many fluorescent compounds are known that are not concerned with the luminescent reaction.

That Luminodesmus, like most other luminous forms, must have oxygen in order to maintain full intensity of light production, was demonstrated by the use of nitrogen and carbon dioxide. Individual animals were placed in the bottom of a cotton-stoppered U-tube through which gases were drawn. The luminescence of animals in an atmosphere of pure nitrogen rapidly faded so that after five minutes only a dim glow was perceptible. This minimum light production was maintained as long as animals were observed (three hours). During this time the animals were quiescent, but apparently the effect on luminescence was specific, for the introduction of oxygen to the U-tube immediately restored full and brilliant light, while the animals returned to normal, unimpaired activity. Whether or not the dim light given off during prolonged exposure to nitrogen also requires small amounts of molecular oxygen remains to be investigated (*cf.* Harvey, 1940, p. 127).

Atmospheres of pure CO_2 were found to cause the rapid and complete disappearance of luminescence within two minutes and also to be extremely toxic. Animals exposed to the gas for 15 minutes not only failed to luminesce but were completely inactivated. Re-introduction of oxygen to the U-tube caused a partial return of light production almost immediately, but that this exposure to CO_2 was close to the lethal point was demonstrated by the fact that some animals failed to recover complete luminosity and activity, and ultimately died. Whether or not CO_2 has a specific effect on the luminescent system is yet to be demonstrated.

Animals were subjected to HCN in a collecting tube strong enough to kill Hymenoptera (Apis) almost instantaneously. The millipedes showed a marked decrease in activity at the end of an hour but no decrease in luminescence below the normal brilliant level. At the end of approximately three hours the animals were completely inactive (dead?) and the luminescence greatly reduced. Neither activity nor full luminescence ever returned when these animals were placed in an atmosphere of pure oxygen. These observations suggest that cyanide is relatively non-toxic to the luminescent system and does not affect it *in vivo* until the lethal point is reached. This is of interest in view of the demonstration of McElroy and Strehler (1949) that HCN not only fails to inhibit luminescence in luminous bacteria and in isolated biochemical systems but actually stimulates light production.

Finally it may be asked what the function of the luminescence is in Luminodesmus. It would appear that it cannot serve as a recognition factor, since all members of this millipede order are blind. As will be seen, Luminodesmus, like many other millipedes, gives off an offensive odor. Cook (1900) says "this absence of eyes also renders apparently meaningless the fact recorded by Bruner and Kenyon that

the repugnatorial secretion of a Nebraska species" (*Fontaria luminosa* Kenyon) "is luminous . . . but when the nocturnal habits of the animals are considered, phosphorescence may be looked upon as affording a protection additional to that of the odor of the repugnatorial fluid."

This hypothesis that a "warning luminescence" has evolved in these millipedes can only be proved by the most carefully controlled field experiments involving nocturnal predators; at the present time it must be said that the role the light plays in the life of the animals is problematical.

Release of HCN

The release of HCN by millipedes has been noted by a number of authors in many species (Guldensteeden-Egeling, 1882; Verhoeff, 1928; etc.).

When collecting live Luminodesmus at the type locality and many times thereafter in handling experimental animals, observers have noted a powerful "cyanide jar" smell. It seemed advisable, therefore, to test for the presence of cyanide. Accordingly, the highly specific and delicate test developed by Fox (1934) was employed. Air is drawn through a system to be tested and then bubbled through an alkaline silver iodide suspension. If HCN is present, the KCN formed immediately dissolves the AgI, the cloudy bluish suspension disappears, and the resulting solution is perfectly clear.

If air was drawn over soil in which the millipedes had been living, a negative test resulted, but air drawn over a single animal which had been slightly agitated in a test-tube gave a strong positive reaction, indicating the release by the animal of HCN.

A biological test demonstrated the poisonous effects of the material released by the millipedes. Bees (Apis) were enclosed in test-tubes with millipedes and slightly agitated. Within three minutes the bees completely lost motor control and if enclosed thus for more than 15 minutes failed to recover. Control bees showed no diminution of angry activity if enclosed in similar stoppered tubes for half an hour.

No direct physiological connection between the phenomenon of luminescence and the release of HCN in Luminodesmus has been demonstrated. Kenyon (1893) and Cook (1900) discuss the possibility that in *F. luminosa* the repugnatorial glands are also the source of the "bead-like" luminosity. It would be of great interest to determine whether the glands of this animal, like those of Luminodesmus and other polydesmoids, release HCN and whether they are in fact involved in the luminescence, particularly in view of the demonstration of McElroy and Strehler, cited above, that cyanide stimulates the light-emitting reaction in other organisms.

Summary

1. The characteristics, distribution and habitat of the millipede, *Luminodesmus sequoiae* Loomis and Davenport are discussed. The life-history and the development of the male gonopods are described.

2. Luminescence is continuous, under no voluntary control, and first appears on hatching; its source apparently lies in the deeper layers of the integument.

3. Extracts of Luminodesmus glow briefly and go out. Experiments to elicit a light flash from such extracts with combinations of Photinus lanterns, magnesium

ion and adenosine triphosphate, and to demonstrate classical "luciferin-luciferase" activity have been without success. The integument of Luminodesmus gives a brilliant yellow-green fluorescence in ultra-violet light.

4. Nitrogen atmospheres reversibly extinguish luminescence, carbon dioxide irreversibly so, while cyanide has little effect on luminescence until the lethal point is reached. Chemical and biological tests have indicated that Luminodesmus, like other millipedes, releases HCN.

LITERATURE CITED

Bruner, Lawrence, 1891. Phosphorescent myriopods. *Insect Life*, 3: 319–321.

Causey, Nell B., 1943. Studies on the life history and the ecology of the hothouse millipede, *Orthomorpha gracilis* (C. L. Koch, 1847). *Amer. Mid. Nat.*, 29: 670–682.

Cook, O. F., 1900. Camphor secreted by an animal (Polyzonium). *Science*, 12: 516–521.

Fox, Denis L., 1934. The silver iodide test for hydrocyanic acid. *Science*, 79: 37.

Guldensteeden-Egeling, C., 1882. Ueber Bildung von Cyanwasserstoffsäure bei einem Myriopoden. *Pflüger's Arch. Physiol.*, 28: 576–579.

Harvey, E. Newton, 1940. Living light. Princeton Univ. Press.

Kenyon, F. C., 1893. A preliminary list of the myriopods of Nebraska, with descriptions of new species. *Proc. Neb. Acad. Sci.*, 3: 14–18.

Loomis, H. F., 1933. Egg-laying habits and larval stages of a milliped *Arctobolus marginatus* (Say) Cook, native at Washington. *J. Wash. Acad. Sci.*, 23: 100–101.

Loomis, H. F., and Demorest Davenport, 1951. A luminescent new xystodesmid milliped from California. *J. Wash. Acad. Sci.*, 41: 270–272.

McElroy, William D., and E. Newton Harvey, 1951. Differences among species in the response of firefly extracts to adenosine triphosphate. *J. Cell. Comp. Physiol.*, 37: 83–89.

McElroy, William D., and Bernard L. Strehler, 1949. Factors influencing the response of the bioluminescent reaction to adenosine triphosphate. *Arch. Biochem.*, 22: 420–433.

Miley, Hugh H., 1927. Development of the male gonopods and life history studies of a polydesmid millipede. *Ohio J. Sci.*, 27: 25–41.

Strehler, Bernard L., and William D. McElroy, 1949. Purification of firefly luciferin. *J. Cell. Comp. Physiol.*, 34: 457–466.

Verhoeff, K. W., 1928. Myriopoda, Diplopoda I. *In* (Dr.) H. G. Bronn's Klassen und Ordnungen des Tier-reichs, Band V, Abteilung II, Buch II, pp. 1028–1030.

THE RELATION BETWEEN OLFACTORY RESPONSE AND RECEPTOR POPULATION IN THE BLOWFLY

V. G. DETHIER

Department of Biology, The Johns Hopkins University, Baltimore 18, Md.

In the course of an investigation to determine rejection thresholds of the blowfly, *Phormia regina* Meigen, for the vapors of homologous alcohols it became desirable to ascertain whether or not the response elicited depended entirely upon stimulation of the olfactory sense, as commonly believed, or represented instead a less specific action by way of a common chemical sense. In order to check this point it was necessary to measure accurately the threshold concentration for some test substance in the vapor phase and then to follow quantitatively changes in threshold value as different areas suspected of bearing olfactory receptors were extirpated. Execution of this plan showed not only that rejection was mediated almost entirely through the olfactory sense, but it yielded in addition some unexpected information relative to the comparative sensitivity of the different olfactory receptor fields and the importance of bilateral versus unilateral stimulation. This last is of particular interest if one is to give due consideration to the possibility of summing and to the relative contributions of the peripheral and central components of the nervous system to those aspects of behavior which depend initially upon stimulation of the sensory system.

Methods and Results

By analogy with other insects, the principal areas to be suspected of bearing olfactory receptors are the antennae, the labial palpi, and the labellum. Frings (1941) had shown by conditioning experiments that the olfactory sense of a related species, *Cynomyia cadaverina* Desvoidy, is resident in the antennae and labellum. Experiments by Dethier and Chadwick (1947) in another connection had shown that the response of *Phormia* to the odors of many organic compounds is abolished when the antennae and labellum are removed. The present investigation was designed to establish these results on a quantitative basis and to assess the contribution of the several sensory areas to the rejection response. Pentanol was selected as a test compound because of the decisive manner in which flies responded to it and the exactness with which a threshold value could be measured. The technique of threshold measurement has been described in detail in an earlier communication (Dethier and Yost, 1952).

For the present study, measurements of response were made with normal animals and animals from which head appendages had been removed by cutting, singly or in various combinations. Instead of varying the concentrations and determining what strength of stimulus was required to elicit 50% response (median rejection threshold) under each experimental condition, the simpler procedure was adopted of selecting two concentrations and determining the change in the per cent of the

TABLE I

Response of normal and operated Phormia to the vapor of pentanol

Receptor areas remaining	Mean % rejection (1)	Mean % rejection (2)	t value	Probability	Significance of difference
Tested at 5.0×10^{-4} *M*					
(1) All (normal) (2) Blank (pure air: no stimulus)	93.3	13.0	23.0	<0.01	+
(1) All except 1 ant. (2) Blank	69.3	11.8	11.3	<0.01	+
(1) Labellum and palpi (2) Blank	47.2	13.8	5.2	<0.01	+
(1) Palpi (2) Blank	34.5	12.7	3.5	<0.01	+
(1) None (2) Blank	15.7	3.0	2.8	0.025	slight
(1) All (normal) (2) All except 1 ant.	93.3	69.3	4.2	<0.01	+
(1) All except 1 ant. (2) Labellum and palpi	69.3	47.2	2.8	0.014	slight
(1) Labellum and palpi (2) None	47.2	15.7	4.4	<0.01	+
(1) Labellum and palpi (2) Palpi	47.2	34.5	1.6	0.15	−
(1) Palpi (2) None	34.5	15.7	2.6	0.02	slight
Tested at 1.6×10^{-4} *M*					
(1) All (normal) (2) Blank	32.3	6.6	4.9	<0.01	+
(1) Labellum and palpi (2) Blank	11.3	11.2	0.012	>0.9	−
(1) None (2) Blank	10.9	10.0	0.21	0.84	−
(1) All (normal) (2) Labellum and palpi	32.3	11.3	2.8	0.017	slight
(1) Labellum and palpi (2) None	11.3	10.9	0.052	>0.9	−

population responding as the various appendages were removed. Since the relation between the per cent of any population responding and the concentration to which it responds is known (the distribution is normal with reference to the logarithm of concentration), this method of approach is justified. The two concentrations selected were 5.0×10^{-6} *M* pentanol, to which approximately 90% of the population normally responds, and 1.6×10^{-6} *M*, to which approximately 25% normally responds. The median is 2.2×10^{-6} *M*.

The results, together with tests of significance, are listed in Table I.

Discussion

Three points for consideration emerge from an inspection of the foregoing data. These relate to the location of receptors, the differential sensitivity of receptor fields, and the difference between unilateral and bilateral thresholds.

When the higher concentration of pentanol is employed as a test stimulus, response in the form of rejection by the animal is abolished only when all of the head appendages (*i.e.*, antennae, palpi, and labellum) are destroyed. On statistical grounds there is still a slight possibility of a retention of some residual sensitivity even then to this concentration (see Table I, "Tested at 5.0×10^{-6} *M*, No Receptors Remaining"), but the degree of response is very low. The reduction of response is most marked following removal of the antennae. No difference in response could be detected between animals possessing labella and palpi only and those possessing palpi only. Therefore, receptors sensitive to the lower concentrations of pentanol are presumed to be present on the antennae and palpi.

This conclusion is in conflict with those previously reported by Dethier and Chadwick (1947) and Frings (1941). Part of the discrepancy may be resolved by giving attention to the problem of stimulus concentration. When a weak stimulus is employed for testing, response is completely abolished following removal of antennae alone (Table I, "Tested at 1.6×10^{-6} *M*, Labellum and Palpi Remaining"). In the earlier experiments with *Phormia*, where the responses of normal versus antennectomized-labellectomized flies were compared, it had been observed that the latter failed to respond to the odors of solutions employed in stimulating tarsal chemoreceptors. The test odors, although not quantitatively controlled at that time, were of low concentration, and a repetition of the experiments has subsequently shown that antennectomy alone is sufficient to prevent the olfactory response of flies under those conditions of testing.

The data for *Phormia* and those obtained by Frings with *Cynomyia cadaverina* could be reconciled by an explanation which would take advantage of the species difference, but this is admittedly unsatisfactory. On the other hand, it is conceivable that the differential sensitivity of the various appendages in the two species is not identical. If receptors sensitive to stimulation by compounds in the vapor phase were considered to be present on all three sets of appendages, the comparative sensitivity in the two species might be antennae > labellum > palpi in *Cynomyia* and antennae > palpi > labellum in *Phormia*. If the stimulus were of moderate to low concentration, only the antennae and labellum would be involved in the first species and the antennae and palpi in the second. Knowledge of such relationships would emerge only after a careful study of the entire concentration range. The fact remains, nonetheless, that *Phormia* is repelled by low concentra-

tions of gaseous substances only when both antennae are intact and by high concentrations only when the antennae or palpi are intact. Therefore repellents in the vapor state may be presumed to be acting on olfactory receptors.

Differences between responses at high and low concentrations of pentanol show quite forcefully that all receptor fields are not equally sensitive and that the field on the mouthparts is much less so than that on the antennae. Additional confirmation for this last conclusion is seen in the degree to which response is reduced following removal of different appendages. The greatest reduction follows bilateral antennectomy. This confirms what has been known for some time, that the antennae of insects are generally sensitive to lower concentrations of odors than other receptor fields. Whether the difference is to be ascribed to a difference in the number of receptors involved, in their intrinsic thresholds, or in their central nervous connections cannot be known at this time.

The possibility of a dependence of total response upon activation of a threshold number of receptors has not been carefully examined in olfaction. The phenomenon has been investigated to some degree for the humidity sense. The suggestion that a threshold number of receptors is required for a response to humidity changes was first demonstrated by Pielou (1940) on evidence gathered with the mealworm, *Tenebrio molitor*. Detailed confirmation was provided by the experiments of Roth and Willis (1951a) which showed that the per cent of response of a population of two species of *Tribolium* was closely correlated with the number of sensilla basiconica remaining on each individual after surgical operation. As the number was progressively decreased the per cent response to humidity differences correspondingly decreased.

The present data on olfactory response to high concentrations of pentanol permit an extension of the analysis of the relation between receptor number and threshold of response. The existence of such a relation is indicated by the significant difference in response between those flies possessing both antennae and those with a single antenna. Observation of individual flies, plus the fact that the response of flies with a single antenna could be made to match the response of normal flies by adjustment of the stimulating concentration, indicated that results could not be attributed to interference with locomotory behavior.

If one makes the assumption that x number of receptors must be activated to insure a response, then at the median threshold value 50% of the flies have x or more receptors acting. The probability at this concentration that a fly (x or more receptors) will be in the half of the population which is responding is 50%. This probability can be increased either by increasing the concentration or by increasing the number of available receptors (n). If n is doubled, the probability of a response increases. This increase can be calculated from the expression $1\text{-}q^2$ where q is equivalent to the per cent not acting at the median concentration. Accordingly, the concentration which elicits a response from 50% of the flies possessing a single antenna should elicit a 75% response from the flies possessing both antennae if there is no interaction between the two antennae.

Although the concentration chosen to test this hypothesis was the 90% level rather than the 50% level, use of the expression $1\text{-}q^2$ still yields the desired results. Application of the test to the data shows that the decrease in threshold with two antennae as opposed to one can be accounted for satisfactorily on a probability basis.

Since the probability of a response can be increased (reflected as a decrease in threshold concentration) either by increasing the receptor number or by increasing the concentration and holding the receptor number constant, it may indeed be concluded that some relation exists between concentration and number of receptors activated. Furthermore, it can be said that as the concentration is increased a larger number of receptors is activated. Beyond this one cannot go on the basis of these data.

TABLE II

Effects of unilateral and bilateral operations in the responses of beetles given a choice between 0% and 100% R.H. at 27° C.
(Data from Roth and Willis, 1951b)

Species	Receptor areas remaining on antenna	% response
Rhyzopertha dominica (thin-walled sensilla present on segments 8 to 10)	Segments 1–10 on both (normal)	76 ± 2.7
	Segments 1–10 on one / Segments 1–9 on other	69 ± 3.0
	Segments 1–9 on both	56 ± 3.4
	Segments 1–10 on one / Segments 1–7 on other	61 ± 2.9
	Segments 1–7 on both	−0.1 ± 7.0
Latheticus oryzae (thin-walled sensilla present on segments 7 to 11)	Segments 1–11 on both (normal)	87 ± 3.1
	Segments 1–11 on one / Segments 1–10 on other	73 ± 3.2
	Segments 1–10 on both	46 ± 2.7
Tribolium castaneum (thin-walled sensilla present on segments 9 to 11)	Segments 1–11 on both (normal)	80 ± 1.0
	Segments 1–9 on both	75 ± 2.9
	Segments 1–9 on one / Segments 1–8 on other	78 ± 3.6
	Segments 1–8 on both	31 ± 5.4
Tribolium confusum (thin-walled sensilla present on segments 7 to 11)	Segments 1–11 on both (normal)	86 ± 1.7
	Segments 1–11 on one / 0 segments on other	76 ± 3.0
	0 antennae	−0.2 ± 2.2
	Segments 1–7 on both	23 ± 1.6
	Segments 1–7 on one / Segments 1–6 on other	12 ± 3.9
	Segments 1–6 on both	5 ± 4.1
	Segments 1–11 on one / Segments 1–6 on other	75 ± 2.1
	Segments 1–6 on both	5 ± 4.1

Rise of threshold with unilateral extirpation has not been emphasized before, but it is implicit in many recorded data. It is seen, for example, in the humidity responses of many beetles. Several selected data of Roth and Willis (1951b) show it clearly (Table II). Here the response is always (with the one exception in the case of *Tribolium castaneum*) reduced when one antenna or portion thereof is removed. The response is further diminished when corresponding areas on the remaining antenna are removed. In none of these cases, nor in the olfactory data presented, does the experimental value of response to bilateral stimulation exceed that for unilateral stimulation by a value greater than that which can be accounted for by statistical bias. Consequently, it can be stated that there is here no evidence

for contralateral neural summation (*cf.* Dethier, 1950). However, when only one or two concentrations rather than a complete frequency distribution have been investigated, it is not possible to analyze the situation fully. Nor can one apply a rigorous test of significance to the difference between the experimental value for bilateral stimulation and the calculated value and consequently settle the question of summation with any great degree of certainty. This follows in part because the Chi square test is inaccurate when small numbers are used and only one degree of freedom is involved.

Since decrease of unilateral threshold over bilateral threshold has not been recorded as occurring commonly among animals (the difference between monaural and binaural thresholds, and monocular and binocular thresholds in man being two of the better known examples), the phenomenon is worthy of closer scrutiny. Additional experiments with the tarsal chemoreceptors, which are more amenable to analysis, are in progress to settle this point. At the moment it would appear that the results reported here do not indicate the existence of true neural summation but instead what has been termed supplementation by Smith and Licklider (1949). Nonetheless, insofar as interaction exists between bilaterally placed receptor fields, it remains from the point of view of the integrated organism a behavioral summation of no little importance.

Summary

1. Measurements have been made of thresholds of rejection of two concentrations of pentanol vapor by normal blowflies and those from which various head appendages have been removed. The concentrations selected were 5.0×10^{-4} *M* pentanol, to which 90% of the population normally responds, and 1.6×10^{-4} *M*, to which approximately 25% of the population normally responds.

2. The results show that the response elicited depends upon stimulation of specialized receptors present on the antennae and palpi and hence cannot be attributed to the action of a common chemical sense. Repellents in the gas phase undoubtedly act as olfactory stimuli. Different receptor fields function at different levels of sensitivity. The antennae are the most sensitive and the various mouthparts less so. There is also a relation between threshold and the number of receptors functioning. Insects possessing both antennae respond to lower concentrations of odor than those bearing only one. This indicates that the two sides of the body are acting in concert. Whether it represents true neural contralateral summation or is based upon statistical bias cannot yet be decided with certainty although the data strongly suggest the latter. In either case, from the point of view of coordinated behavior it illustrates the manner in which bilaterally placed receptor fields may operate as a unit.

LITERATURE CITED

Dethier, V. G., 1950. Central summation following contralateral stimulation of tarsal chemoreptors. *Fed. Proc.*, **9**: 31–32.

Dethier, V. G., and L. E. Chadwick, 1947. Rejection thresholds of the blowfly for a series of aliphatic alcohols. *J. Gen. Physiol.*, **30**: 247–253.

Dethier, V. G., and M. T. Yost, 1952. Olfactory stimulation of blowflies by homologous alcohols. *J. Gen. Physiol.* (in press).

FRINGS, H., 1941. The loci of olfactory end-organs in the blowfly, *Cynomyia cadaverina* Desvoidy. *J. Exp. Zool.*, **88**: 65–93.

PIELOU, D. P., 1940. The humidity behaviour of the mealworm beetle, *Tenebrio molitor* L. II. The humidity receptors. *J. Exp. Biol.*, **17**: 295–306.

ROTH, L. M., AND E. R. WILLIS, 1951a. Hygroreceptors in adults of Tribolium (Coleoptera, Tenebrionidae). *J. Exp. Zool.*, **116**: 527–570.

ROTH, L. M., AND E. R. WILLIS, 1951b. Hygroreceptors in Coleoptera. *J. Exp. Zool.*, **117**: 451–488.

SMITH, M. H., AND J. C. R. LICKLIDER, 1949. Statistical bias in comparisons of monaural and binaural thresholds: binaural summation or binaural supplementation. *Psychol Bull.*, **46**: 278–284.

THE DIGESTION AND ABSORPTION OF FAT IN LAMELLIBRANCHS [1]

W. C. GEORGE

Department of Anatomy, University of North Carolina, Chapel Hill, North Carolina

The problem of digestion in lamellibranch mollusks has been a subject of interest to biologists for many years. It seems well established that the digestion of carbohydrates takes place largely extra-cellularly in the lumen of the stomach through the action of enzymes liberated through the dissolution of the crystalline style. The place and mode of digestion and absorption of proteins and fats have occasioned disagreement. One concept supported especially by a series of papers by Yonge (1923, 1926a, 1926b, 1930, 1946) holds that the digestion of fats and proteins is exclusively or largely intra-cellular, occurring in phagocytes of the blood and in cells of the digestive diverticula. Another group of investigators has questioned the correctness of this view and has presented some evidence to indicate that the digestion of fats and proteins occurs extra-cellularly through the action of enzymes of uncertain source (Swano, 1929; Nelson, 1933; Mansour, 1945, 1946; Mansour-Bek, 1945, 1946).

The present author's concern with the structure and primitive functions of blood and hemolymph gives the problem of digestion in invertebrates a special interest. The thesis of intra-cellular digestion in leucocytes was appealing and tentatively accepted (George, 1941). That digestion by phagocytes of the blood was so extensive as to be a significant feature of the digestive process did not appear soundly enough established, however, to justify its acceptance as a basis for generalization. The present investigation, which is limited to the digestion and absorption of fats, was undertaken during July–August, 1950, to determine the extent of participation of leucocytes in the digestive process of some lamellibranchs.

Material and Methods

Several species of lamellibranchs were used but principally the common oyster, *Crassostrea virginica*, and the ribbed mussel, *Modiolus demissus*, both abundantly available in Beaufort Harbor. Stained neutral fats (olive oil and peanut oil) were fed as emulsions stained with Sudan black B or with Sudan III (Scarlet red). Scarlet red,[2] which stains both neutral fats and fatty acids pink, was chosen for most experiments because of its advantage in subsequent counter-staining with Nile blue sulfate. Emulsions of the oil, previously stained to saturation, were prepared as follows: 60 cc.

[1] This investigation was aided by a grant from the Carnegie Foundation to the University of North Carolina. It was carried out in part at the Beaufort, N. C., laboratory of the United States Fish and Wild Life Service during the summers of 1950 and 1951. Thanks are due to Mr. Albert C. Day, Director of the Service, for permission to use the facilities of the laboratory and to Mr. Clinton Atkinson, Director of the laboratory.

[2] The scarlet red used in this investigation was given to the author a few years ago by the late Simon Henry Gage and is from the lot used by him and Fish in their investigations.

of the stained oil, 15 grams of powdered acacia and 30 cc. of distilled water were put in a Waring Blendor and thoroughly mixed. The emulsion was then thinned somewhat by the gradual mixing in the Blendor of 20 cc. additional distilled water. This produced a satisfactory stock emulsion with particles ranging in size from less than 1 μ to perhaps 10 μ. Oil immersion examination of a diluted drop of the stock emulsion shows the larger droplets to have a clear pink color; a slight but definite pink is recognizable in medium sized droplets; no definite color is detectable in individual droplets of the smallest size. In some cases the emulsion was inserted with a pipette through the mouth into the stomachs of animals after the removal of one valve. Sometimes animals were fed in salt water aquaria. A few drops of the emulsion poured into the water, kept turbulent by bubbling air through it from an air compressor, is quickly distributed throughout an aquarium. As some of the oil slowly accumulates at the surface, this can be skimmed off and the concentration restored by the addition of fresh emulsion.

Fat droplets withdrawn with a pipette from the stomachs at definite times after feeding, or droplets taken from other experimental material can be identified by color when examined with the microscope. By counter-staining with a solution of Nile blue sulfate allowed to run under from the edge of the cover glass, one can determine if any hydrolysis of neutral fat has occurred. Droplets that have undergone reduction to fatty acid are changed from pink to a clear blue as the Nile blue sulfate reaches them, whereas droplets of neutral fat become deeper pink (an orange pink). The same microscopic picture is obtained after exposure of emulsion to a solution of commercial lipase (steapsin).[3] No droplets of the untreated stock solution turn blue when counter-stained with Nile blue sulfate; on the contrary they all turn a deeper pink. As a counter-stain, concentrations of Nile blue sulfate ranging from 1:1000 to 1:300,000 were used and proved effective. A solution of 1:10,000 was settled on as most satisfactory.

Tests for lipase in styles and various other structures were made by mixing small quantities of stock emulsion with a mince of the structures in 2–3 drops of sea water or tap water on a hollow ground slide and subsequently counter-staining a sample with Nile blue sulfate. Generally no bactericidal agent was used; similar results were obtained when a drop of 0.5% phenol solution was added to 2 drops of water of the mince.

Material for sections was fixed in 10% formalin in sea water.

Results

Oysters and mussels kept for as long as five days in turbulent water with a suspension of olive oil emulsion stained with Sudan black showed no deposition of stained fat in any of the storage tissues although the emulsion was freely taken into the gut. Similar experiments with emulsions of peanut oil stained with scarlet red likewise gave no evidence for storage of stained fat such as Gage and Fish (1924) found in mammals and birds.

Although these experiments gave no evidence for storage of experimental fat in the tissues, the evidence for digestion within the stomach seems clear. In emulsion withdrawn and tested after having remained in the stomach for only a short time, some droplets became a pale blue after Nile blue counter-staining, indicating the

[3] Lipase (steapsin) from Nutritional Biochemicals Corporation was used.

beginning of fat digestion, but many droplets retained the pink color of neutral fat. In emulsion that had remained in the stomach for several hours, the number of blue droplets after Nile blue sulfate was much greater and the depth of blue was deeper, most drops showing an intense blue color. In emulsion withdrawn and tested after a still longer time in the stomach, some of the larger droplets had a shrunken appearance, due, perhaps, to the diffusion of glycerol from the drops; and after 30 hours exposure to stomach juice some droplets contained needle-like crystals, probably fatty acid crystals.

My findings with regard to the digestion of fat in the stomach seem to be in conflict in some essential matters with the conclusions of Yonge (1926b), who fed olive oil stained red with Nile blue sulphate to oysters. On p. 351 he says, "In the lumen of the stomach there were immense numbers of phagocytes, most of them with ingested oil. In certain cases they collected in great numbers round large droplets of oil, which had turned blue under the influence of their enzymes. *All oil droplets lying free in the stomach and not surrounded by phagocytes retained the red color*—evidence of the absence of lipase in the stomach." The presence of phagocytes and their ingestion and digestion of oil I can confirm; but the absence of digestion of oil other than under the influence of phagocytes is not in accordance with my observations. It is conceivable, of course, but not likely that *Ostrea edulis* and *Crassostrea virginica* differ markedly in their physiology of fat digestion and absorption, but it seems more probable that the oil observed by Yonge had been inadequately exposed to the action of stomach juices.

If the disintegration of leucocytes liberates appreciable amounts of lipase or if they secrete it, it seems reasonable to assume that some should be present in the plasma. To test for lipase in plasma, fat emulsion diluted with sea water was injected into the hearts of several oysters and mussels and one clam. In all cases, tests for fat digestion after two to ten hours were positive so far as digestion within blood cells was concerned and negative for extra-cellular digestion in plasma. Also, hanging drop cultures of fat emulsion mixed with blood were made. Examination after one hour showed ingestion and digestion of pink fat droplets within leucocytes. No evidence for fat digestion could be found in the droplets that remained in the plasma. Further tests for lipase in plasma were made during August, 1951, by placing drops of plasma from fresh water mussels, salt water mussels and oysters upon smears of margarine, unstained peanut oil, and Sudan III-stained cotton seed oil. This was to check the technique and results of Yount (1950), who reported the digestion of neutral fat by the plasma of oysters, mussels and clams. My several experiments were negative, no droplet ever turning blue after Nile blue except in the case of one batch of oil stained with Scarlet red the previous summer and carried over. In smears from that lot of oil, some blue droplets appeared after counter-staining with Nile blue. It turned out, however, that blue droplets appeared outside the areas to which plasma was applied as well as inside them and, indeed, in smears to which nothing was applied except Nile blue. Evidently that particular batch of oil was rancid, and it seems possible that Yount's positive results were due to rancid fat in his margarine. These observations seem to establish two points: 1) the leucocytes produce their own lipase, and 2) there is no evidence that they liberate any into the plasma (or, presumably, into stomach juice).

To determine the source of the lipase of the stomach juice, a drop of fat emulsion was added to minced crystalline style in two or three drops of sea water. Without

exception these tests were positive for lipase activity. The specimens tested consisted of 12 oysters, 15 mussels, one clam (*Venus mercenaria*), one *Atrina livida*, and one razor clam (*Ensis directus*).

Two typical case reports from my notes will illustrate the progress of digestion. *Mussel MA*—style was removed, washed, and minced in 2 drops of sea water; a drop of emulsion was added at 9:15 A.M. At 11 A.M. tests with Nile blue sulfate showed 10–15% of oil droplets to be blue; at 12 M. about 25%; at 2:30 P.M. almost 100%. *Oyster 27-B*—style minced in sea water at 11:45 A.M. At 12:30 P.M. 75% of the droplets are blue after Nile blue; at 4:45 P.M. 100% are blue. There is variation among individual oysters and mussels in the speed of the digestive action, or in the percentile response of fat droplets. These experiments indicate that in all these species of lamellibranchs the style is an important source of lipase.

To determine if active lipase was present in the digestive diverticula, fat emulsion was mixed with mince of a chunk of the glandular mass in two or three drops of sea water. Tests with Nile blue sulfate were made after varying times with the following results: all 9 oysters tested and the single *Atrina rigida* were negative for fat digestion; all 10 mussels tested and the single clam and the razor clam were positive. In the case of one mussel, the lining membrane of the stomach and the glandular mass were dissected apart, minced and tested separately. The glandular mass was positive and the lining membrane was negative for lipase. Although the tests for fat digestion by mince of the digestive diverticula were clearly positive in some species, they were less strongly so than was the case with the styles. The conclusion seems justified, then, that the principal source of lipase for extra-cellular digestion in the stomachs of the lamellibranchs studied is in the crystalline styles. Apparently this source may be supplemented in some species with lipase secreted by glandular tissue of the digestive diverticula.

Observations were made to determine if fat is transported from the lumen of the gut into the tissues by free phagocytes as supported by Yonge (1926b) and Takatsuki (1934). It seems reasonable to expect to find some if they are to be looked upon as significant agents in digestion and absorption. Oysters that had been kept in a suspension of stained fat emulsion in sea water were sacrificed at 8, 18, 21, 22, 25, 29, and 30 hours. Considerable fat was in the guts of these animals. Careful examination revealed no leucocytes with ingested experimental fat when blood was withdrawn from the hearts. In most specimens a few cells were found with apparent oil droplets, but no pink color was identifiable. These droplets stain blue with Nile blue, as do fatty acids (and intrinsic granules of oyster blood cells). In the connective tissue surrounding the gut epithelium, free cells with globules of a probably fatty nature were present but in no case could it be determined that they contained experimental fat. On the basis of the evidence one could say only that the blood cells may possibly transport a small amount of fat into the blood and tissues but the amount of transport is not enough to be significant in total nutrition.

Is the neutral fat used in these experiments absorbed at all and if so, how? To get an answer to these questions, sections of stomachs of experimental and control oysters were made by the freezing technique. Examinations of unstained sections from animals fed fat emulsion showed abundant small granules or droplets in the ciliated epithelium of the stomach and in the ciliated epithelium of the ducts of the digestive diverticula. Under high powers of the microscope these droplets individually show no color; but under low power they give a diffuse pinkness to the epi-

thelial layer. There is little doubt that they are from the stained fat fed the animals. A secondary staining of the section with an alcoholic solution of Sudan III colors these droplets a bright orange. Counter-staining with Nile blue sulphate colors them a deep blue. The conclusion seems justified that they are droplets of the stock emulsion reduced to fatty acid and in process of being absorbed through the epithelium. After secondary staining with Nile blue or Sudan III it is clearly seen that the droplets are most abundant in the apical one-third to one-half of the columnar epithelial cells and least abundant in the middle third, a distribution accounted for in part, at least, by the fact that the nuclei occupy a large part of the middle third of the epithelial layer. Cells of some areas of the mucosa contain more droplets than other areas.

Frozen sections of control animals, neither fed nor starved, showed some sudanophil droplets in the epithelium but no such great accumulation as in the case of the animals fed oil.

What appear to be blood cells are to be found sometimes in the epithelium of both experimental and control animals. They are not present in sufficient numbers, however, as to justify corroboration of the conclusions of Yonge (1926b) and Takatsuki (1934) that wandering phagocytes are important agents of absorption in the gut. On the contrary, the facts from these experiments seem to lead to the conclusion that neutral fat fed to these lamellibranchs is reduced to fatty acid in the lumen of the gut and absorbed as independent particles through the ciliated epithelium of the gut and ducts of the digestive diverticula, and that little if any is carried through by leucocytes.

The epithelium of the tubular alveoli of the digestive diverticula shows two types of cells which tend to be associated in groups: 1) cells with coarse to fine granules of greenish brown color, and 2) smaller cells with smaller colorless granules of approximately uniform size, which Yonge (1926a) considers to be immature cells. Neither of the types of granules in these cells stains with alcoholic Sudan III. After Nile blue sulfate the greenish-brown granules become deep green; the colorless granules found in the smaller cells do not stain. No fat droplets could be discovered within or between the epithelial cells of the tubular alveoli as were found in the ciliated epithelium of their ducts and of the stomach. Hence, the results of feeding emulsified oil to oysters provide no evidence for the ingestion or absorption of fat particles by the epithelium of the digestive tubules.

What evidence do these experiments reveal concerning the storage of fat in the connective tissue around the gut? The connective tissue of lamellibranch mollusks contains large polygonal vacuolated cells with centrally located nuclei having a zone of perinuclear cytoplasm with strands radiating outward to a zone of peripheral cytoplasm. The vacuolar spaces within these cells contain few or many droplets of various sizes which in unstained sections are colorless. Sudan III in 70% alcohol colors them bright orange; Nile blue sulfate colors them blue. They are dissolved by higher alcohols and xylene. They probably contain stored fatty acid, but in unstained frozen sections of experimental animals no pink color could be detected in these droplets to identify them as having been derived from the stained emulsion fed the oysters. In addition to the fixed vacuolated cells of the connective tissue, large wandering cells are present. They contain drop-like inclusions of different sizes ranging in color from a clear brown to a pale color that might be mistaken for pink. They contain in addition small, relatively uniform

granules resembling the intrinsic granules of cells of the blood. Droplets and granules both stain a blue green with Nile blue sulfate but they do not stain with alcoholic Sudan III. They are not dissolved by alcohols and xylene, and they are present in animals not fed fat emulsions as well as in those that are. It is suggested that these inclusions are not neutral fat or fatty acid but probably chromolipoids, perhaps mixed with other substances.

Discussion

Evidence from this investigation for the extra-cellular digestion of fats in the stomachs of oysters and mussels seems unequivocal and conclusive. Previous evidence has been contradictory. Claude Bernard (1855) reported that the stomach juice of oysters digests starches and fats. Albrecht (1921) found that extracts of the intestinal tracts of the clam, *Tivella stultorum*, have lipolytic action. More recently Mansour-Bek (1945) reported lipolytic activity by digestive juice pipetted from the stomachs of *Tridacna elongata* and *Pinctata vulgaris*. On the other hand, Yonge (1926a, 1926b, 1930, 1937, 1946) supports the thesis that in lamellibranchs digestion of proteins and fats is exclusively intra-cellular, the only extra-cellular enzymes normally present being those set free when the head of the crystalline style is dissolved in the stomach. He finds these enzymes to be exclusively amylolytic (Yonge, 1923, 1926b). Any proteolytic or lipolytic action found in filtered stomach juice is attributed to cytolysis of phagocytes. In this view he is supported by Graham (1931). (Other pertinent references are to be found in the papers cited above.)

Correlated with the differences of opinion in regard to intra-cellular and extra-cellular digestion in lamellibranchs is a corresponding lack of harmony concerning the enzymatic activity of the crystalline style and of the digestive diverticulum. It has become well established that the dissolution of the style liberates amylolytic enzymes. With regard to other enzymes the observations of workers are at variance. Yonge in a number of papers contends that the style consists largely of amylolytic enzymes and that no other enzymes are present in it. Chestnut (1949) with the methods he used found amylolytic but no demonstrable proteolytic or lypolytic activity in extracts of the crystalline style. Some other investigators get results not entirely in accord with Yonge's. Sawano (1929) reported the presence of lipase as well as amylase and protease in extracts of the crystalline style. Fox and Marks (1936) also attributed lipase as well as carbohydrases to extracts of the style of *Mytilus*. In discussing the digestion of animal forms by the oyster, Nelson (1933), without specifying the nature or the source of the active substances, states that "It is evident therefore that some substance or substances present in the stomach of an oyster with a well formed style can penetrate the chitin of crustacea and the cuticle of nematodes resulting in death and disintegration of the animals." Mansour (1946) and Mansour-Bek (1946) seem to consider that globules pinched off or shed from cells of the digestive gland are the source of extra-cellular lipolytic and proteolytic enzymes.

The experiments and observations reported in a previous part of this paper have an important bearing on certain features of these arguments. Sensitive tests show that hydrolysis of neutral fats does occur extra-cellularly in the cavity of stomachs of oysters and mussels. These tests also give a positive reaction for the presence of

lipase in aqueous extracts of the crystalline style, which appears to be the principal source of the enzymes that mediate the hydrolysis of fat in the stomach. It is possible that lipolytic enzymes from the digestive diverticulum supplement those from the style, since minced diverticulum in water gives a positive reaction for the presence of lipase in individuals of some species, not all. One should not overlook the possibility, however, that normally this demonstrated lipase from the digestive gland might never become an extra-cellular enzyme but function intra-cellularly.

It is still an open question whether the tubules of the digestive gland are secretory or whether they exclusively absorb and digest phagocytically. A detailed exploration of the functions of the digestive gland was not a part of this investigation. However, some observations are significant. We have seen that the gland contains, possibly secretes, lipase in some species, but none was demonstrated in the oysters of these experiments. Chestnut (1949), working with the same species of oyster, found that extracts of the digestive diverticula have lipolytic activity as well as amylolytic and proteolytic activity. This difference in results suggests the possibility of seasonal variation in the presence of active enzymes or perhaps a difference in the effectiveness of different techniques in extracting enzymes from the cells. Phagocytic action is suggested by the fact that cells of the tubules contain what appear to be bits of foreign debris and greenish-brown granules of an organic nature that may have been ingested but probably were manufactured. These experiments produced no positive evidence and little circumstantial evidence that cells of the tubules ingest fat emulsion. It could be that the tubules of the digestive diverticula are specialized for the phagocytosis or absorption of some substances, not of others. Many authors have attributed an absorptive function to them. Saint-Hilaire (1893) found that the cells of the tubules of some lamellibranchs and other mollusks are not ferment cells but absorptive cells. Enriques (1902) and Gutheil (1912) consider that they absorb but that their main function is excretory. List (1902) found that in *Mytilus* particles of India ink were taken from water containing it by cells of the digestive diverticula. Vonk (1924) got similar results with oysters. From microscopic examinations of glands during the later stages of digestion in *Ostrea* and *Modiolus*, Nelson (1925) was led to believe that in these forms even more food is digested in the digestive glands than in the stomach. Yonge (1926a), by feeding iron saccharate to various lamellibranchs, found that the substance was taken up by amoebocytes and by cells of the tubules of the digestive diverticula but by no other part of the gut. Coe and Fox (1944) state that in *Mytilus californianus* "Many of the smallest objects and particles are phagocytized by the cells lining the digestive diverticula."

As regards the processes of absorption of fat, the observations here reported indicate that it is largely absorbed as particulate matter through the ciliated epithelium of the gut and the ducts of the digestive diverticula. None was identified in the cells of the tubules themselves. All fat observed in the process of being absorbed gives the reaction for fatty acid. There is no evidence for or against absorption as soluble fat, although it seems reasonable to assume that the glycerol component is so absorbed. Although leucocytes free in the lumen of the gut may ingest fat droplets in quantity, the evidence does not indicate any considerable transport of fat by them into the tissue since no leucocytes with identifiable experimental fat could be found in the blood or tissues. Furthermore, in confirmation of the ob-

servations of others, in both oysters and mussels I have observed many leucocytes in the lumen of the gut with ingested diatoms; but I have never seen any in the blood or tissue spaces, nor, so far as I am aware, has any one else. However, Gutheil (1912) figures and describes blood cells with food balls passing from the gut into the connective tissues. Yonge (1926b) did report that cells with ingested fat moved back into the blood spaces and tissues in *O. edulis*. I cannot confirm it for *C. virginica*. Wandering cells with lipoidal inclusions characteristically present in the connective tissues may have misled observers. As pointed out elsewhere, these lipoidal inclusions in *C. virginica* differ from the experimental fat in certain respects: they stain blue-green instead of blue with Nile blue sulphate; they do not stain with alcoholic Sudan III, and they are insoluble in absolute alcohol and xylene.

In spite of the evidence for digestion and absorption of stained emulsified fat, why was no evidence obtained in regard to the deposition of stained fat in the tissues of oysters and mussels? Gage and Fish (1924) got clear-cut results with birds and mammals and were able to follow stained fat through two generations. Three possible explanations come to mind: 1) oysters and mussels may be unable to re-synthesize the fatty acids of olive oil and peanut oil into their own specific fat, since the fatty acids of these oils differ from the unsaturated fatty acids of plankton on which the lamellibranchs feed (Markley, 1947); 2) fat may be deposited in connective tissue cells in such small discrete units that the pink color of the droplets is not distinguishable under the microscope, and the total amount of fat in an area may not be massive enough to be detectable on gross examination; 3) at the time of these experiments the stained fat absorbed may have been rapidly metabolized rather than stored. At certain periods, during the summer or after the development of spawn or fattening in the fall, fat is apparently rapidly metabolized. Nelson (1951) reports that great quantities of fat and oil can be demonstrated in New Jersey oysters in late spring before the development of spawn, and in late summer and early fall prior to the "fattening" which follows summer spawning. He suggests that this may be preliminary to the rapid build-up of glycogen, in which the storage in the gland of substantial quantities of oil constitutes the first step. This point deserves further investigation.

Summary

Emulsions of olive oil and peanut oil stained with Sudan stains were fed to oysters and mussels and mixed with mince of crystalline styles and other structures. Samples of the experimental material were subsequently examined to determine if the fat was absorbed and how, and counterstained with Nile blue sulphate to determine if the neutral fat had been split to fatty acid and glycerine. The following observations and conclusions resulted:

1. Free droplets of neutral fat are hydrolyzed in the stomach.
2. Droplets of the stained fat in the form of fatty acid appeared in large numbers in the ciliated epithelium of the stomach and ducts of the digestive gland. None was found in the non-ciliated epithelium of the alveoli of the digestive gland.
3. Droplets of the emulsion were ingested by leucocytes in the lumen of the gut. Neutral fat in leucocytes was hydrolyzed. There was no certain evidence of the passage of any leucocytes with ingested fat back into the blood spaces or tissues.

4. Emulsion injected into the cavity of the heart or mixed in hanging drops of blood was ingested by leucocytes. Hydrolysis occurred within blood cells. No evidence was found for the hydrolysis of neutral fat by the plasma.

5. Tests of stained emulsion subjected to the influence of minced styles showed hydrolysis of neutral fat in all cases.

6. Emulsion subjected to the influence of minced digestive gland was positive for the hydrolysis of neutral fat in some species, but not in others.

7. No evidence for deposition of stained fat in the tissues was found. Possible explanations are suggested.

LITERATURE CITED

ALBRECHT, P. G., 1921. Chemical studies of several marine mollusks of the Pacific coast. *J. Biol. Chem.*, **45**: 395–405.

BERNARD, CLAUDE, 1855. Leçons de physiologie experimentale appliqué à la médécine. Paris.

CHESTNUT, A. F., 1949. Studies on the digestive processes of *Ostrea virginica*. Ph.D. thesis (unpublished) Rutgers University. A brief abstract is in *Jour. Elisha Mitchell Sci. Soc.*, **65**: 215–216 (1949).

COE, W. R., AND D. L. FOX, 1944. Biology of the California sea-mussel (*Mytilus californianus*). III. Environmental conditions and rate of growth. *Biol. Bull.*, **87**: 59–73.

ENRIQUES, P., 1902. Il fegato dei Molluschi e le sue funzioni. *Mitth. Zool. Neapel.*, **15**: 281–407.

FOX, D. L., AND G. W. MARKS, 1936. The digestive enzymes (food of *Mytilus californianus*). *Bull. Scripps Oceanog. Inst. U. Cal. Tech.*, **41**: 5–11.

GAGE, S. H., AND P. A. FISH, 1924. Fat digestion, absorption and assimilation in man and animals as determined by the dark-field microscope, and a fat-soluble dye. *Amer. J. Anat.*, **34**: 1–85.

GEORGE, W. C., 1941. Comparative hematology and the functions of the leucocytes. *Quart. Rev. Biol.*, **16**: 426–439.

GRAHAM, A., 1931. On the morphology, feeding mechanisms, and digestion of *Ensis siliqua* (Schumacher). *Trans. Roy. Soc. Edinburgh*, **56**: 725–752.

GUTHEIL, F., 1912. Über den Darmkanal und die Mitteldarmdrüse von *Anodonta cellensis*. *Zeitschr. Wiss. Zool.*, **99**: 444–538.

LIST, T., 1902. Die Mytiliden. *Fauna und Flora d. Golfes v. Neapel*, v. 27.

MANSOUR, K., 1945. The zooxanthellae, morphological peculiarities and food and feeding habits of the Tridacnidae with reference to other lamellibranchs. *Proc. Egypt. Acad. Sci.*, **1**: 1–11.

MANSOUR, K., 1946. Food and digestive organs of lamellibranchs. *Nature*, **158**: 378.

MANSOUR-BEK, J. J., 1945. The digestive enzymes of *Tridacna elongata*, Lamk., and *Pictata vulgaris*, L. *Proc. Egypt. Acad. Sci.*, **1**: 13–20.

MANSOUR-BEK, J. J., 1946. Extra-cellular proteolytic and lipolytic enzymes of some lamellibranchs. *Nature*, **158**: 378–379.

MARKLEY, K. L., 1947. Fatty acids. Interscience Press, New York.

NELSON, T. C., 1925. Recent contributions to the knowledge of the crystalline style of lamellibranchs. *Biol. Bull.*, **49**: 86–99.

NELSON, T. C., 1933. On the digestion of animal forms by the oyster. *Proc. Soc. Exp. Biol. Med.*, **30**: 1287–1290.

NELSON, T. C., 1951. Personal correspondence.

SAINT-HILAIRE, C., 1893. Sur la fonction du foie des Crustacé et des Mollusques. *Revue Sc. St. Petersbourg*, **4**: 93–117.

SAWANO, E., 1929. Studies on the enzymes of *Ostrea circumpicta*. *Sci. Rep. Tohoku Imp. Univ., ser. 4 (Biol.)*, **4**: 327–334.

TAKATSUKI, S. I., 1934. On the nature and functions of the amoebocytes of *Ostrea edulis*. *Quart. Jour. Micro. Sci.*, **76**: 379–431.

VONK, H. J., JR., 1924. Verdauungsphagocytose bei den Austern. *Zeitschr. f. Vergleich. Physiol.*, **1**: 607–623.

YONGE, C. M., 1923. Studies on the comparative physiology of digestion. *J. Exp. Biol.*, **1**: 15–63.

YONGE, C. M., 1926a. The digestive diverticula in the lamellibranchs. *Trans. Roy. Soc. Edinburgh*, **54**: 703–718.

YONGE, C. M., 1926b. Structure and physiology of the organs of feeding and digestion in *Ostrea edulis*. *J. Mar. Biol. Assoc.*, **14**: 295–386.

YONGE, C. M., 1930. The crystalline style of the mollusca and a carnivorous habit cannot normally co-exist. *Nature*, **125**: 44–45.

YONGE, C. M., 1937. Evolution and adaptation in the digestive system of the metazoa. *Biol. Rev.*, **12**: 87–115.

YONGE, C. M., 1946. Digestion of animals by lamellibranchs. *Nature*, **157**: 729.

YOUNT, J. L., 1950. A study of the blood of some marine lamellibranchs. Master's thesis (unpublished), University of North Carolina.

THE EFFECT OF INGESTED RADIOPHOSPHORUS ON EGG PRODUCTION AND EMBRYO SURVIVAL IN THE WASP HABROBRACON [1, 2, 3]

DANIEL S. GROSCH AND ROBERT L. SULLIVAN

Genetics, Biological Division, N. C. State College of Agriculture and Engineering of the University of North Carolina, Raleigh, N. C.

The present report is the first of a series concerned with investigations of the developmental and genetic effects of ingested radioactive isotopes. Although an appreciable amount of information is becoming available on the vertebrate histopathology of internal radioactive isotopes, apparently there have been few studies directed toward the cytogenetic consequences. Even in such organisms as insects, which produce large samples of offspring in a relatively short time, available data are limited to a few short papers on Drosophila (King, 1949; Bateman and Sinclair, 1950; Blumel, 1950).

The insect chosen for present investigations, an ectoparasite of Ephestia caterpillars known in genetic literature as *Habrobracon juglandis* (Ashmead), has the Hymenopteran advantage for induction experiments of parthenogenetically produced, genetically haploid males. Thus the haploid egg and the organism developing from it can indicate the effects of experimental treatment without the complications of another set of chromosomes. As will be detailed in "Materials and Methods," particular advantages were discovered in the use of Habrobracon for feeding experiments.

The purpose of the following experiments was to evaluate among embryonic offspring the total induced lethality, including recessive lethals, dominant lethals and non-nuclear damage, for a series of dosages ranging from tracer dilutions up into the intermediate category of radioactivity encountered in medical therapy (Levy, 1946). Phosphorus, the element chosen, is well represented in nucleic acids which are intimately associated with the structural elements of cell nuclei. Incidental observations on the longevity of treated animals are included, which indicate a remarkable radio-resistance of adult wasps.

Materials and Methods

The Habrobracon stock used was wild type number 33 known at least 15 years for its high hatchability (Whiting, 1940). Unmated females were stored in clean shell vials (70 × 20 mm.) for five days in an incubator at 30° C. Such conditions

[1] This document is based on work performed under Contract AT-(40-1)-1314 for the Atomic Energy Commission.

[2] Appreciation is expressed for the facilities of the Marine Biological Laboratory, Woods Hole, Mass. and for the extremely helpful advice of Dr. Norman A. Baily of the M.B.L. Dept. of Experimental Radiology and the Columbia University Radiological Research Laboratory.

[3] Published with the approval of the Director of Research, N. C. Agr. Exp. Station as paper No. 409 of the Journal Series.

of starvation produce a hungry individual with an abdomen very obviously flattened dorsoventrally. The visible change in abdominal shape reflects a utilization of reserves stored in the fat body and the disappearance of all but a few full-sized uterine eggs (Grosch, 1950). At the end of the fifth day a droplet of white clover honey with or without P^{32} was delivered to the wall of the vial from the end of a straightened No. 3 paper clip. Typically the hungry wasp is attracted to the honey within one minute, and, standing upon clean surface, contacts the honey only with the mouth parts. This is a distinct advantage over experimenting with insects which move over the surface of a radioactive medium when feeding. The gonads of an insect with such "browsing" habits are constantly near an external source of irradiation and the interpretation of results is thereby complicated. We believe that ingestion experiments with Habrobracon most nearly approach an evaluation of the effects of internal radiation alone. Note further that only one feeding was used per female.

During feeding, the flattened abdomen gradually swells until its fullness approaches that of an unstarved wasp. The re-attained rotundity is due entirely to the filling of an expansible crop situated in the anterior of the abdomen. This is a second advantage, because by mere external inspection the actuality and degree of feeding can be determined.

The braconid digestive tract extends in direct course from mouth to anus with the crop an enlarged, thin-walled connection between the esophagus and stomach. It is the structure known in the honey bee as the honey stomach. It is not a diverticulum as in other orders of insects and within a day its contents are moved into the midgut, yet the crop with its strong anterior and posterior valves can serve in efficient immediate storage of a significant volume of ingested food (Grosch, 1951). The capacity of the crop furnishes a defined volume basis for dosage in feeding experiments (a third advantage). The mean volume for distended crops from undwarfed adult females was determined as 0.49 ± 0.06 mm^3.

When the crop is completely filled, the wasp loses interest in feeding. The majority of Habrobracon females attain satiety at one continuous feeding although a few individuals seem to prefer intermittent ingestion. Because of the latter type of behavior, the standard procedure adopted was to allow a given female one hour in the vial with the food droplet. At the end of such a period the great majority of wasps are completely fed. This was determined by an inspection of the degree of distension of the abdomen.

Twelve hours after the single honey feeding each female was given two pre-stung caterpillars on which to deposit eggs. Stender dishes (60×35 mm.) large enough for spreading out the 10 caterpillars parasitized by 5 females were the containers used at this phase. Caterpillars rested upon white paper fitted into the bottom of the dishes. A daily renewal of one caterpillar per female has been adopted as the standard procedure. If additional caterpillars appeared discolored or unduly flaccid, they were replaced and a notation made on the data sheet. Such precautions are believed necessary to avoid introducing variables due to the condition of the host.

Eggs were collected every 24 hours and immersed in mineral oil (Nujol) contained in Syracuse watch glasses, a procedure determined as giving hatchability records even better than eggs left on the host. After 48 hours incubation in mineral oil at 30° C., the number of larvae which had hatched was counted and recorded. In our experience, since 30 hours is the normal hatching time at 30° C., all larvae which can emerge will have done so in 48 hours.

In all but the first experiment a weight basis was used for the preparation of samples of honey containing P^{32} at various activities. To this end, small (36 × 12 mm.), thick-walled bottles equipped with plastic screw caps were weighed before and after adding pipetted amounts of honey and diluted radioactive phosphorus in the approximate proportions needed to obtain a desired activity. In setting up the initial experiment it had been discovered that the forces of adhesion and cohesion negated pipetted delivery of exact volumes of honey. Dosages mentioned below are given in terms of the activity at the time of ingestion. The complicated question of internal dosage, although under consideration, has not progressed to a state which would warrant usage in the present paper.

Records upon the laying and hatchability were taken for 42,832 eggs, of which 24,832 were controls. Table I presents a numerical summary of experiments to indicate their scope and sample size. Also shown is the number of females used in each experiment. The analysis presented in the section on "Results" is a consideration on an average-per-animal basis.

TABLE I

A summary indicating the size of ovipositing groups and the total number of eggs laid by each group

Experiment number	Experimentals			Controls	
	Activity of material ingested in μc/g.	Number of females	Eggs laid	Number of females	Eggs laid
5	1,445	22	498	19	3,496
6	1,070	22	604	19	3,010
1	1,000–	19	769	21	3,379
2	271	17	816	18	2,377
3	166	20	2,671	20	3,152
4	43	16	2,471	17	1,820
7	20.6	20	2,264	17	2,684*
9	16.99	24	4,640	24	4,914
8	12.6	16	2,854	17	2,684*
		176	17,587	155	24,832

* The same control was used for two simultaneous experiments, numbers 7 and 8.

RESULTS

The gametic sequelae of treatment internally with P^{32} were found to have organization on the basis of the treatment's influence upon egg production. That is, egg laying (1) may or (2) may not be halted comparatively early in the life of a treated female. A dose adequate to halt egg production is about 200 μc/g. At lower doses, egg production continues throughout the life of the female. However, two types of effects are observed: (a) Between doses of 50 to 200 μc/g. there is definite falling off in egg production followed by recovery; while (b) below 50 μc/g. no statistically significant decrease in egg production is demonstrable.

(1) *Egg laying halted.* Figure 1 depicts the average day by day egg production for females fed sufficient radioactive phosphorus to cause early cessation of egg laying. Presumably at massive dosages, still fewer eggs would appear in an

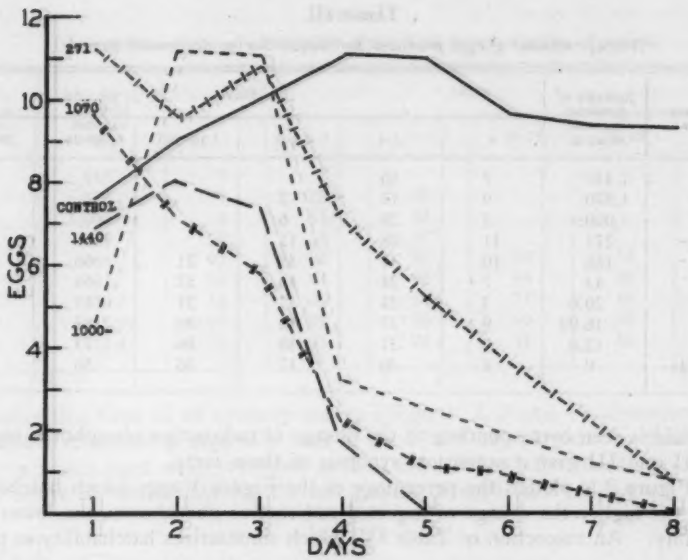


FIGURE 1. A comparison with combined controls of the average number of eggs produced per female per day by Habrobracon which ingested radioactive phosphorus sufficient to halt egg production. The low number of eggs produced in the first day of the 1000-experiment is non-significant; the individual control showed a similar value. The designation at the beginning of each curve is the activity in millicuries per gram of the mixture fed.

approach to the limit of those eggs differentiated before treatment and un-resorbed during a five day starvation period. On the present graph an orderly relation is noted with the highest dosage causing earliest discontinuation of egg laying and the least number of eggs laid. At successively lower dosages a sequence in number

TABLE II

Cumulative total of average number of eggs produced per female

Experiment number	Activity of mixture ingested in μc/g.	Day 1	Day 4	Day 9	Day 19	Day 29	Day of death
5	1,445	7	23				23
6	1,070	9	26	28			28
1	1,000—	5	30	36			36
2	271	11	39	50			50
3	166	10	47	82	103	169	206
4	43	7	31	74	126	190	192
7	20.6	3	27	64	91	140	158
9	16.99	9	36	76	174	258	269
8	12.6	2	29	67	153	226	249
Controls	0	8	38	85	161	217	238

TABLE III

Average number of eggs produced per female during designated periods

Experiment number	Activity of material ingested in μc/g.	Days					
		1	2–4	5–9	10–19	20–29	29–death
5	1,445	7	16				
6	1,070	9	17	2			
1	1,000—	5	25	6			
2	271	11	28	11			
3	166	10	37	35	21	66	37
4	43	7	24	43	52	64	2
7	20.6	3	24	37	27	49	18
9	16.99	9	27	40	98	84	11
8	12.6	2	27	38	86	73	23
Controls	0	8	30	47	76	56	21

of eggs laid is seen corresponding to the dosage of radioactive phosphorus ingested. Tables II and III give a numerical synopsis of these facts.

On Figure 2 is plotted the percentage of the Figure 1 eggs which hatched. In general, the higher the dosage of ingested radioactive phosphorus, the lower is the hatchability. An inspection of Table IV, which summarizes hatchability in periods

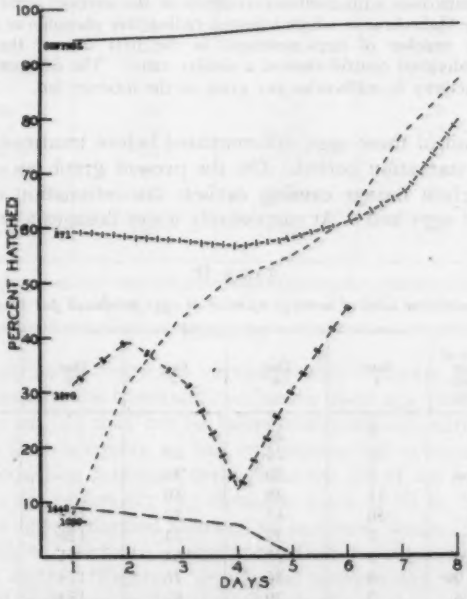


FIGURE 2. The hatchability of the eggs of Figure 1. The activity in millicuries per gram of the mixture fed is again indicated at the beginning of each curve.

TABLE IV

Average percentage of hatchability of eggs laid during designated periods

Experiment number	Activity of material ingested in μc/g.	Days					
		1	2–4	5–9	10–19	20–29	29–death
5	1,445	9	7				
6	1,070	9	22	28			
1	1,000–	8	44	67			
2	271	60	58	67			
3	166	93	87	85	52	53	63
4	43	98	97	96	95	80	50
7	20.6	51	83	84	75	57	45
9	16.99	96	97	96	94	87	—
8	12.6	59	91	94	91	80	62

selected on the basis of all primary curves (Figs. 1, 2, 3 and 4), demonstrates the hatchability relationship especially well in the 2 to 4 day period. In those experiments in which eggs were produced several days beyond the fourth day, there is shown a rise in hatchability for the last groups of eggs laid. However, it should

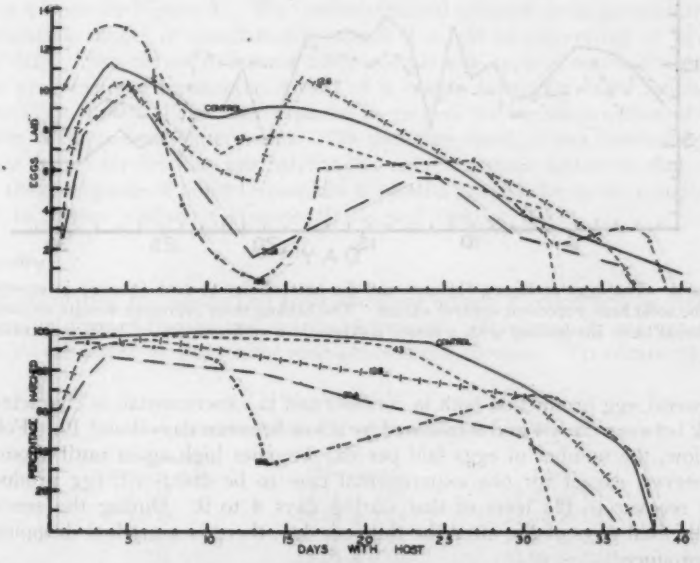


FIGURE 3. A comparison with combined controls of the eggs laid per female per day (above) and the hatchability thereof (below) in experiments when eggs are produced throughout the life of treated females. Curves are smoothed after the thirtieth day. The activity in millicuries per gram of the mixture fed is indicated along the middle of each curve where space is more adequate rather than at the beginning of the curve when the activity was at the level designated.

be remembered that these samples are quite small and the viability of even a single egg markedly affects the numerical result.

(2) *Egg laying not halted.* Figures 3 and 4 depict eggs laid and the hatchability thereof for experiments in which egg production continued throughout life. The results plotted in Figure 4 were treated separately to avoid adding confusion to already complex drawings (Fig. 3). Furthermore, unlike all other experiments, in the investigations for Figure 4, egg laying and hatchability were not recorded after the thirty-first day of oviposition. (In actuality, records beyond the thirty-first day would have been insignificant additions to the data obtained since only one animal survived the thirty-second day.)

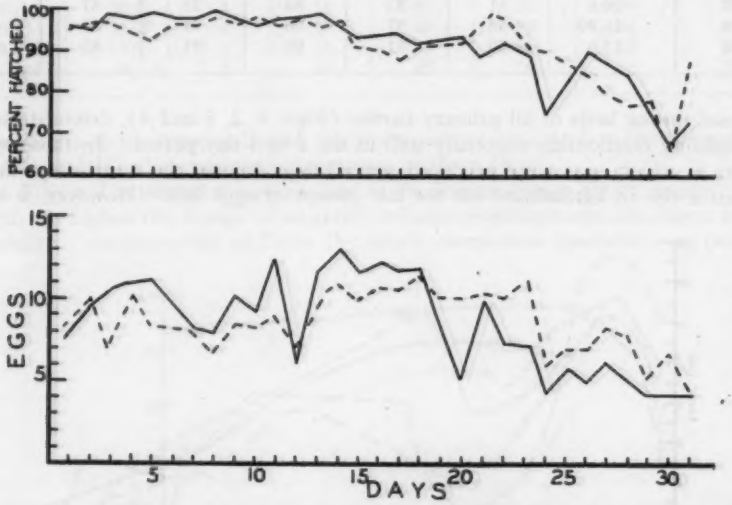


FIGURE 4. The eggs produced (below) and the hatchability thereof (above) in experiment No. 9. The solid lines represent control values. The broken lines represent results on an average per animal basis for feeding with a honey mixture at a radioactivity of 16.99 millicuries per gram.

In general, egg production both in controls and in experimentals is characterized by a peak between days 4 and 9 followed by a low between days 9 and 19. Following this low, the number of eggs laid per day becomes high again until about day 29. However, except for one experimental case to be discussed, egg production does not recover to the level of that during days 4 to 9. During the senescent period, the final days of life after the thirtieth day, there is a gradual dropping off in eggs produced.

As is shown in Table II higher doses tend to result in fewer eggs. When plotted graphically this consequence of treatment is not strikingly evident except throughout the course of days 9 to 19. During this period the drop in egg production is much accentuated in higher dosages. However, after day 19 the production of eggs by treated and untreated wasps becomes similar so that curves approach

each other and differences are not significant. Table III furnishes a numerical summary by designated periods of eggs laid.

The results observed when the number of emerged larvae is compared with the number of eggs laid are rendered in percentages and plotted in Figures 3 and 4. The resultant curves are not as complex as those for egg production. Except for the highest dosage in this group (166 μc/g.) no obvious depression was demonstrated in the graphs.

In general, until day 29, the experimental curves parallel the curve for controls but at a lower value indicating a measure of lethality. After the twenty-ninth day the differences between controls and experimentals are statistically non-significant. In fact, in unsmoothed plots from raw data the lines cross and recross almost at daily intervals. Again it should be remembered that the samples of eggs obtained become small in the last few days.

Survival curves

A popular method of representing the results of radiation experiments is by means of the survival curves obtained through plotting the proportion of organisms surviving against the dose. This gives a picture of the manner in which the number of organisms affected increases with the dosage of radiation. In the present case, the values of the first three designated periods shown in Table IV have been used as a basis for Figure 5. The curves obtained promise to be particularly useful by indicating ranges of irradiation in which it would be interesting to have additional data. The curves indicate a more complicated type of action than the geometric progression (exponential curve) of a system involving single ionizations of targets (Lea, 1947). This was expected in view of the inclusive nature of induced lethality in the present experiments. On the other hand, it was interesting to find that our curves for first day egg hatchability exhibit a shape similar to that obtained when the emergence of adult Drosophila is plotted against the initial activity of P^{32} added to culture medium (Arnason, Irwin and Spinks, 1949).

Longevity

By calculating the means and their standard errors, a conception is obtained of the survival time of treated and untreated females. These results are presented in Table V, calculated as days after emergence from cocoons. To obtain days after

TABLE V

Mean longevity in days of females which have ingested P^{32}

Activity of material ingested in μc/g.	Experimental	Control
1,445	23.14 ± 2.08	27.89 ± 1.35
1,070	24.82 ± 1.74	25.37 ± 1.58
1,000–	21.32 ± 1.81	27.09 ± 2.47
271	25.06 ± 3.09	22.06 ± 2.31
166	26.45 ± 2.16	23.45 ± 2.02
43	26.94 ± 1.83	21.59 ± 2.11
20.6	29.10 ± 2.75	26.94 ± 2.71
16.99	27.17 ± 1.90	27.92 ± 1.85
12.6	28.63 ± 1.96	26.94 ± 2.71
	Combined controls	25.73 ± 0.65

treatment, five days should be subtracted from each value. It is evident from the data obtained that longevity of wasps was not influenced significantly by internal β radiation over the range investigated. In fact, the experimentals show higher mean survival time than the corresponding controls in five out of the nine experiments. In the distribution and range of values which make up the array from which mean survival time is calculated, examples of both treated and non-treated wasps were obtained in the long-lived category of 40 days after the honey feeding. Typically, at

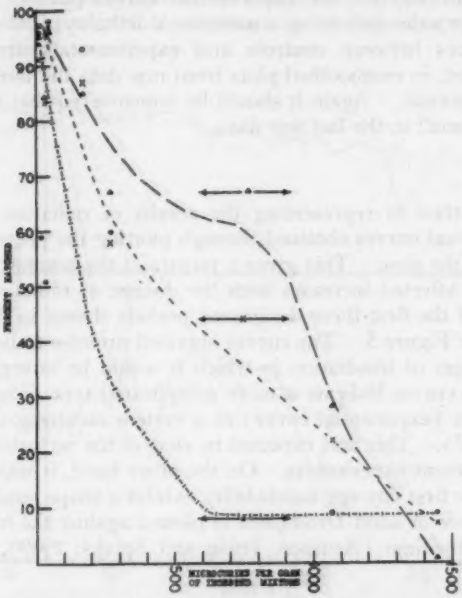


FIGURE 5. Survival curves obtained by plotting the percentage of hatched eggs against the radioactivity of the mixture ingested. The results for the first day of egg laying are designated by ovals. The corresponding curve is drawn of alternating horizontal and vertical dashes. The results for the period comprising the second to fourth day are designated by x marks and the curve is drawn using short dashes. The results of the period comprising the fifth to ninth day are designated with triangles and the curve is drawn using long dashes. The arrows mark the possible range of dosage for the initial experiment when pipetted measurement was attempted.

least three to six animals were alive after 30 days of experimental observation. Such longevity was spectacular in experiments in which egg production was halted five to eight days after treatment, yet the sterilized females remained alive for weeks after laying their last egg. A more comprehensive investigation into what appears to be remarkable radio-resistance by adult braconids is in progress.

DISCUSSION

Interpretation of the above results is difficult because we have not been able to find comparable studies with the day-to-day life-time approach. In general the

total and average egg productivity of controls compares well with the surveys of Hase (1922) and associates who obtained more than 200 eggs per individual braconid collected from nature. However, hatchability records by these European investigators run between 50 and 70 per cent whereas hatchability in the selected laboratory stock used by us and by A. R. Whiting is typically above 95 per cent. Even when the senile period is included the overall average hatchability is at least 80 per cent.

Occasional checks on egg production and hatchability at various ages have been made during exploratory and control studies by A. R. Whiting (personal communication). However these data have not been published nor do they include observations during the senile period. In the published studies with unmated females (Whiting, 1940, 1945) attention is directed chiefly to the first period of oviposition in the interest of comparing the radio-sensitivity of eggs in successive stages of first meiotic pro- and metaphase. Habrobracon furnishes ideal material for such studies and the results throw some light upon certain of our findings.

The braconid ovary is comprised of four ovarioles of the polytrophic type, thus containing alternating successions of oocytes and trophocytes for at least three quarters of each tube's length. The anterior end of each ovariole is made up of mitotically active oogonia subsequently differentiated into cell groups, of which the most posterior cell becomes the functional oocyte while remaining members of the group become trophocytes. As an oocyte is moved posteriorly its nucleus passes through prophase of first maturation division; eggs ready to be deposited are stored in the posterior of each ovariole in first meiotic metaphase. These observations from dissections were correlated with the results of X-radiation experiments (Whiting, 1940) to demonstrate that eggs in first meiotic metaphase were relatively susceptible to radiation damage, whereas oocytes in prophase with diffuse chromatin were relatively resistant. Radio-sensitivity and radio-resistance were reflected by low hatchability and high hatchability, respectively. Such differences in sensitivity could explain the rise in hatchability shown by the present curves during days 6 to 8 wherein the greater percentages hatched of the eggs laid on these days could reflect a resistance of prophase eggs to radiation.

It is not settled whether most of the radiation damage occurs shortly after ingestion or if injury occurs during an appreciable number of successive days. At present we lean toward the former, since it is suspected that much radioactive phosphorus is lost by incorporation into the eggs laid. Although radio-autography of Habrobracon has not yet been performed, it is well established in biological material that the deposition of P^{32} is heaviest in regions of the greatest metabolic and mitotic behavior associated with growth and differentiation. Typically, in the adult holometabolous insect, cell division and differentiation take place only in the gonads. Thus, Irwin, Spinks and Arnason (1950) find that Drosophila gonads continue to show very high P^{32} content in the adult when general growth of all other tissues has ceased. Consistent with these concepts is the present fact that with Habrobracon egg laying prolonged throughout life, experimental results in both egg production and in egg viability ultimately approach control values.

There are certain differences between our results and those of A. R. Whiting especially noticeable in the first day of egg laying on which the number of eggs obtained by us is consistently lower. This is undoubtedly due to the differences in

the conditions of the experiment. The animals of the present experiment were maintained under circumstances which prevent the formation of uterine eggs, whereas the conditions in the Whiting experiments were conducive to the accumulation of uterine eggs.

Our finding a cessation of egg production after subjecting females to higher dosages of internal radiation seems another addition to the several reports of the radio-sensitivity of oogonia in various animals. Four examples have come to our attention: (1) With higher X-ray dosages Whiting (1940) discovered oogonial regions vacuolated, fragile and with pyknotic nuclei attendant to a falling-off in Habrobracon egg production. (2) A case of total absence of ovaries has been reported for female mosquitoes raised in baths containing P^{32} at activities of around 200 mμc. (Bugher and Taylor, 1949). (3) The most spectacular effect of internal radiations in vertebrates is shown in mice injected with P^{32} by reduction in the size of ovaries (Warren, MacMillan and Dixon, 1950). The immediate size reduction following injection is due to degenerative follicular changes; however, a failure to regain normal size is traceable to subsequent greatly diminished oogenesis. (4) Ionizing radiations are found to exert a selective and destructive action on the gonads of round worms (Alicata, 1951). These representative cases from such diverse phyla suggest a universal consequence to be expected in radiation experiments. It seems likely that numerous other examples may exist in published and unpublished data on still other types of animals.

When our observations on unhalted egg laying are considered, for comparison are available the lifetime records of egg laying for birds, especially chickens (Hutt, 1949), and for other insects, especially Drosophila (Gowen and Johnson, 1946). Records for all forms of life show a decline in egg production with age as do the present data. In this effect of senescence, as might be expected, the abilities of Habrobracon more nearly resemble those obtained for dipteran rather than those obtained for avian egg production. This is explainable on the basis that the normal egg production by insects is not markedly influenced by cyclic events. Egg laying as plotted by us for Habrobracon shows smoother day-to-day curves for the first half of the wasp life period than exhibited by eight different races of fruit flies considered by Gowen and Johnson. However, by suggesting two peaks, our curves seem more complicated in their general shape. A current experiment is being performed in an attempt to estimate whether starvation conditions preceding egg laying complicate the egg production curve. If not, an alternative explanation may be based on the change-over from eggs produced by oocytes differentiated before eclosion to eggs produced by oocytes differentiated during the laying period.

Lifetime records of modifications to egg laying, due to experimental effects comparable to ours, have not been discovered in available literature. In order to obtain data somewhat comparable, we have gone to reports concerning the effects of ionizing radiations on cell production, where we find curves which ours resemble. This is so for diagrammatic presentations of the rate of change in peripheral blood cells following graduated dosages of penetrating radiations (Cronkite, 1949). Blood cells are the ultimate products of mitotically active tissues. In turn, this thought suggested another source of comparable curves, those based upon mitotic index. Here, too, we note a resemblance, especially in the shapes of our lines plotted for the period between days 9 and 29 (Fig. 4). These curves are inter-

estingly reminiscent of certain results obtained by Canti and Spear (1929) in irradiated, cultured chick tissue. Similar consequences of radiation have been demonstrated in other rapidly dividing tissues from such diverse origins as beans, rats and tadpoles (Lea, 1947). As measured by division activity, higher dosages result in a drop in cell production with the control level never regained, whereas in intermediate dosages a recovery wave is obtained which exceeds the control level. A present example of a recovery wave exceeding the control may be furnished by the 12.6 μc/g. curve.

An accepted explanation of such recovery waves is that the mode results when two classes of cells arrive at the same stage at a given time. A class of cells not in the susceptible phase at the height of treatment, and hence not greatly influenced, is supplemented by another class of cells which were delayed in division but have recovered. In explanation of our lifelong egg production curves we postulate similar delayed division effects upon the oogonia. It is conceivable that the oogonia forming a mitotically active cell mass would have cells in susceptible phase at the height of the treatment, which could be delayed in division and development.

Summary

1. Changes in the eggs produced per day and in the hatchability thereof are reported for virgin Habrobracon fed honey containing P^{32} at radioactivities ranging from the tracer level into the intermediate level of internal therapy (10 μc to 1500 μc).

2. Above 200 μc/gm. of ingested mixture, egg laying ceased after a period of a few days corresponding to a fraction of the supply of unresorbed ova and oocytes differentiated before treatment. The viability of the embryos from these eggs, as measured by the number hatched, is correlated with the level of activity in the material ingested. A rise in viability in the seventh to eighth day, in experiments in which egg production continued that long, is interpreted as an indication of the lesser susceptibility of prophase eggs.

3. Below 200 μc/gm. of ingested mixture, egg laying continued throughout the life of the treated females; however, with higher dosages fewer eggs were produced. This is traceable chiefly to a decline in the eggs laid daily during the middle period of life. Curves obtained show recovery waves reminiscent of those found for cell production from rapidly dividing tissues after irradiation. Viability of the eggs produced during the first two-thirds of life is correlated with dosage. During the senescent decline in egg production, typical of both controls and experimentals, no significant differences in hatchability were obtained.

4. A remarkable radio-resistance was discovered, in that treated animals in *all* experiments lived as long as or longer than controls.

LITERATURE CITED

Alicata, J. E., 1951. Effects of roentgen radiation on *Trichinella spiralis*. *J. Parasitology*, **37**: 491–501.

Arnason, T. J., R. L. Irwin and J. W. T. Spinks, 1949. Some effects of P^{32} on the development of Drosophila. *Canadian J. Res.*, **27**, Sect. D: 186–194.

Bateman, A. J., and W. K. Sinclair, 1950. Mutations induced in Drosophila by ingested phosphorus 32. *Nature*, **165**: 117–118.

Blumel, J., 1950. The action of radioactive phosphorus in Drosophila. *Science*, **111**: 205–206.

BUGHER, J. C., AND M. TAYLOR, 1949. Radiophosphorus and radiostrontium in mosquitoes. Preliminary report. *Science*, **110**: 146–147.

CANTI, R. G., AND F. G. SPEAR, 1929. The effects of gamma irradiation on cell division in tissue culture *in vitro*. Part II. *Proc. Roy. Soc. London, Ser. B.*, **105**: 93–98.

CRONKITE, E. P., 1949. The hematology of ionizing radiations. Chapt. VIII in Atomic Medicine. Thomas Nelson and Sons, N. Y.

GROSCH, D. S., 1950. Starvation studies with the parasitic wasp Habrobracon. *Biol. Bull.*, **99**: 65–73.

GROSCH, D. S., 1951. The ingestion capacity of the wasp Habrobracon. *Biol. Bull.*, **101**: 222.

GOWEN, J. W., AND L. E. JOHNSON, 1946. Metabolic capacity of different races of *Drosophila melanogaster* for egg production. *Amer. Nat.*, **80**: 149–179.

HASE, A., 1922. Biologie der Schlupfwespe *Habrobracon brevicornis* (Wesmael). *Arb. biol. Reichsanst. Land. u. Forstw.*, **11**: 95–168.

HUTT, F. B., 1949. Genetics of the fowl. Pp. 333–339. McGraw-Hill Book Co., N. Y.

IRWIN, R. L. B., J. W. T. SPINKS AND T. J. ARNASON, 1950. Deposition of P^{32} in developing Drosophila. *Canadian J. Res.*, **28**, Sect. D: 137–142.

KING, R. C., 1949. Genetic changes accompanying the uptake of P^{32} by *Drosophila melanogaster*. *Rec. Gen. Soc. Am.*, **18**: 98–99.

LEA, D. E., 1947. Actions of radiations on living cells. Cambridge Univ. Press.

LEVY, H. A., 1946. Some aspects of the design of radiochemical laboratories. *Chem. Eng. News*, **24**: 3168–3173.

WARREN, S., J. C. MACMILLAN AND F. J. DIXON, 1950. Effects of internal irradiation of mice with P^{32}. Part II. *Radiology*, **55**: 557–570.

WHITING, A. R., 1940. Sensitivity to X-rays of different meiotic stages in unlaid eggs of Habrobracon. *J. Exp. Zool.*, **83**: 249–269.

WHITING, A. R., 1945. Effects of X-rays on hatchability and on chromosomes of Habrobracon eggs treated in first meiotic prophase and metaphase. *Amer. Nat.* **79**: 193–227.

REGENERATION IN ISOLATED AND FUSED PIECES OF CLAVA LEPTOSTYLA[1]

NORMAN E. KEMP

Department of Zoology, University of Michigan, Ann Arbor, Michigan, and Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine

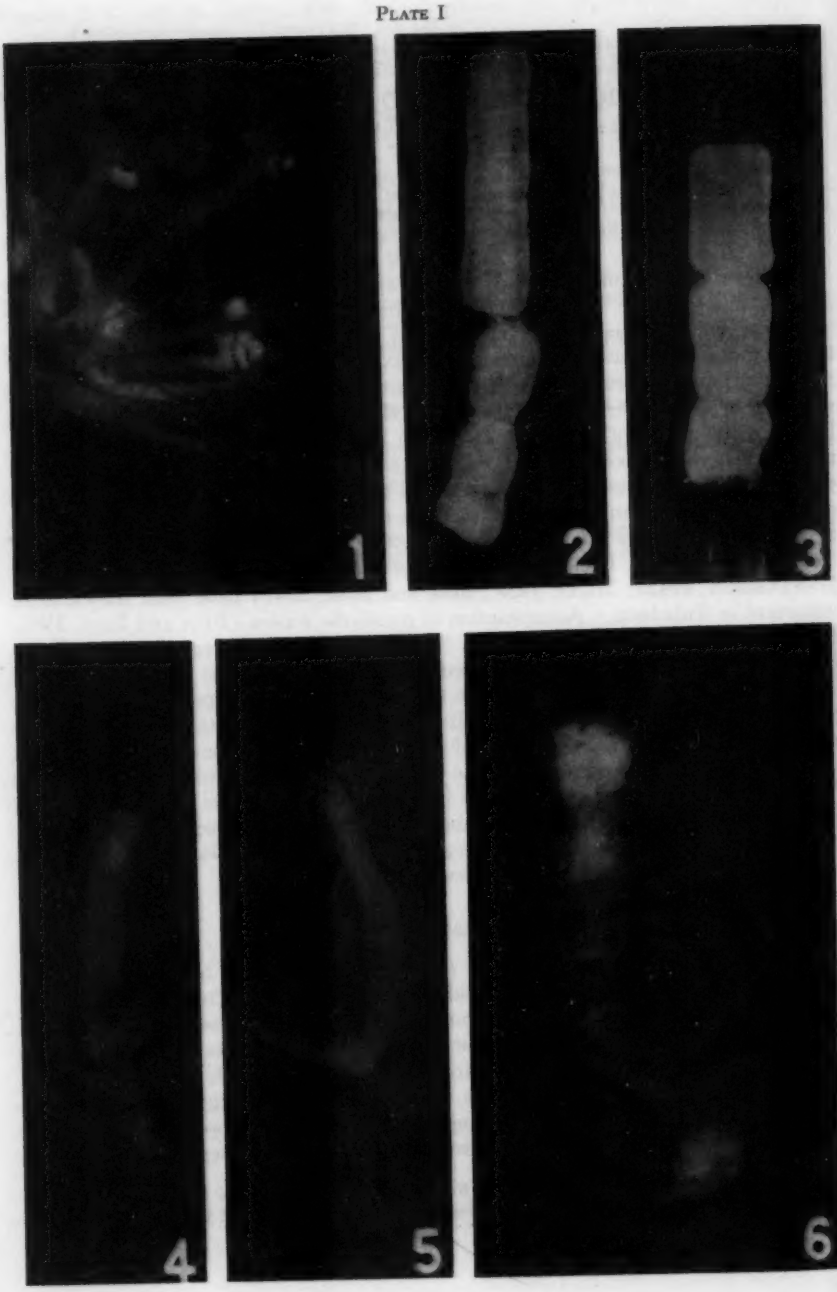
Modern experimental studies on regeneration in hydroids, as Peebles (1931) anticipated, have been aimed at elucidating the factors responsible for this dramatic morphogenetic response. It is well known (reviews of Child, 1929, and Barth, 1940a) that coelenterate hydranths exhibit axial gradients of metabolic activity and that distal levels exert dominance over proximal levels. Barth (1938b) demonstrated that in Tubularia circulation within the gastrovascular cavity is responsible for the maintenance of dominance, for if an isolated stem was ligatured hydranths might regenerate at both ends. The same result was attained if circulation in the coelenteron was blocked with an oil droplet (Barth, 1938b), with a bubble of oxygen or nitrogen or by means of an inserted glass rod (Rose and Rose, 1941). A series of recent papers have considered certain extrinsic factors affecting regeneration. Temperature (Moog, 1941) and the level of oxygen supply (Barth, 1938a, 1940b, 1944; Goldin, 1942b; Miller, 1937; Rose and Rose, 1941) have been shown to be important in Tubularia. Accumulation of metabolic wastes (Rose and Rose, 1941; Miller, 1942) or lowered pH (Goldin, 1942a, 1942b) prevent regeneration. Zwilling (1939) showed that hydranths could be elicited along the side of a stem of Tubularia by cutting a window through the perisarc, thereby exposing the coenosarc directly to sea water, and Goldin and Barth (1941) have investigated the reorganization of coenosarcal fragments free of perisarc. As background for further work on the physiology of regeneration, the present paper deals with the potentialities for regeneration revealed in isolated or fused pieces of hydranths of *Clava leptostyla* Agassiz. Papers by Hargitt (1906 and 1911) contain valuable descriptive information on this species and a more recent paper by Brien (1943) reports experiments on regeneration in a related species, *Clava squamata*.

Materials and Methods

During the summer of 1950 *Clava leptostyla* was found to be abundant on the Fucus attached to rocks in the intertidal zone at Salisbury Cove, Maine. Clava grows in the form of colonies of separate light orange hydranths (Fig. 1) attached to a mat of hydrorhiza firmly adherent to the substratum. Mature polyps, which are about 1 cm. in length when expanded, consist of a contractile stalk, a gonosome, tentacle-bearing region, and hypostome. The gonosome is a region possessing several short, branched gonophores bearing clusters of spherical sporosacs, which are either male or female. There is no free medusoid generation; instead a planula

[1] Aided by a grant from the Horace H. Rackham School of Graduate Studies, University of Michigan, and an Ulric Dahlgren Memorial Fellowship, Mt. Desert Island Biological Laboratory.

PLATE I



larva is released. There is no separate perisarc around the stalk of the hydranth but the perisarc is represented by a thin, non-living surface layer closely adherent to the ectoderm. Pieces to be isolated or fused were cut from individual hydranths with iridectomy scissors. Operations were performed in sea water and pieces were cultured at room temperature in small petri dishes immersed in finger bowls containing approximately one inch of filtered sea water. In early experiments it was noticed that isolated portions of the stalk usually rounded up and failed to undergo regeneration unless one end became attached to the culture dish. Accordingly, it became routine procedure to secure one end of an isolated piece by pinning it with a fine glass rod thrust through the tissue (Fig. 2) and into a substratum of 2% agar. Pieces to be fused were strung on a fine glass rod, then held tightly together for about 12 hours by means of glass tubing slipped over the ends of the rod and held in position with flat sections of cover glass resting on the exposed outer ends of the rod (Fig. 3). Photographs were taken with a Kodak Bantam camera mounted above one ocular of a stereoscopic microscope.

I wish to thank Dr. Maria Anna Rudzinska for directing my attention to the abundance of Clava at Salisbury Cove, and Dr. Philip R. White, in whose laboratory this work was done.

Experiments

A. *Pieces isolated without agar substratum*

The first experiments tried were simple tests of the regenerative behavior of (1) the isolated hypostome and tentacle-bearing region, (2) the gonosome, (3) isolated stalks. Ten days after isolation 7 of the first group had become attached to the glass culture dish and were putting out hydrorhizal protuberances. Twenty-four others which remained unattached showed no basal outgrowths. It was noted by the twentieth day that most of the unattached specimens had undergone dedifferentiation of the tentacles originally present and had not regenerated. Those attached still showed their distal tentacles as well as a regenerated stalk and hydrorhiza. Isolated gonosomes (group 2) showed a marked tendency to regenerate new tentacles and hypostome at the apical end. After 10 days this was true in 8 of 23 specimens examined and one of these had regenerated two new oral ends. None

Figure 1. View of colony of *Clava leptostyla* showing expanded hydranths; gonosome with spherical sporosacs just proximal to tentacle-bearing region.

Figure 2. Specimen illustrating method of pinning with a fine glass rod used to anchor stems to substratum of 2% agar. Here rod is through middle of one member of a fused pair of stalks.

Figure 3. Specimen illustrating method of fusing pieces. Two stalks strung on a single glass rod are being fused end-to-end through pressure exerted by glass tubes slipped over each end of the rod.

Figure 4. An unusual example showing regeneration of apical structures at both ends of a stalk isolated for 15 days. Hydrorhizal outgrowth is seen at level of glass rod through original basal end of stalk; basal hydranth possibly a result of secondary regeneration.

Figure 5. A typical example of primary regeneration showing tentacles at original apical end and hydrorhizum at original basal end of stalk isolated for 15 days; pinned through basal end.

Figure 6. Specimen showing stalk of one hydranth fused between basal ends of stalks of two other complete hydranths. After 19 days no regeneration had occurred at lines of fusion.

of the gonosomal pieces became attached, hence did not develop hydrorhiza. In the third group (isolation of the stalk) many of the specimens developed bulbular enlargements with asymmetrical protuberances at one end, presumably the primordia of hydrorhiza from the original aboral end. Fifteen specimens, all still unattached, had these outgrowths after 10 days, but only 7 of the 15 had regenerated new tentacles. By the twentieth day 4 had become attached. Two of these had regenerated oral structures at the apical end of the stalk and one had a new hydranth growing out from the basal hydrorhiza.

In another group of experiments the regenerative behavior of isolated individuals of Clava in various dilutions of sea water was tested. Dilutions differing by 10% intervals from 0 to 100% sea water were tried. Forty per cent sea water or less proved too hypotonic, but the organisms showed remarkable tolerance in surviving all dilutions down to 50% sea water. Two groups of complete hydranths, isolated in 50% and 80% sea water, respectively, survived during 17 days of observation. It appeared that during this time dedifferentiation of tentacles and gonosome was more rapid than in controls kept in 100% sea water. After dedifferentiation had occurred, regeneration of new hydranths took place. One specimen in 80% sea water had regenerated 8 new hydranths at the end of the 17 days.

The above results and others not reported here indicated that two types of regenerative response should be distinguished: (1) primary regeneration resulting in the differentiation of hypostome and tentacles at the apical end, usually within 3–7 days after isolating pieces of the hydranth; and (2) secondary regeneration, meaning the delayed differentiation of completely new hydranths either from hydrorhizal outgrowths or from dedifferentiated tissue of any part of an old hydranth. The former response undoubtedly depends on the apico-basal gradient already established, while the latter represents establishment of a new center of organization in an undifferentiated primordium. The results indicated also that the mechanical factor of attachment favors both primary and secondary regeneration.

B. *Isolated stalks pinned to substratum of agar*

After the importance of attachment was realized, a series of experiments on isolated stalks was performed. Isolated pieces were pinned with a fine glass rod through one end or through the middle. One end of the rod was then thrust vertically into a substratum of 2% agar in the culture dish. This procedure favored primary regeneration (Figs. 4, 5) since pieces anchored at one end could elongate. They were thus saved from rounding up and degenerating, the usual fate of unanchored stems. The results are recorded in Table I.

TABLE I

Primary regeneration of isolated entire stalks pinned to substratum

Position of pin	No. of animals	Age after isolation	Number regenerating	% regenerating
Apical end	10	7 days	1	10
Middle	19	7 days	12	63
Basal end	15	7 days	11	73

It is seen that there is a distinct difference between specimens pinned through the basal end or middle of entire stalks and those pinned through the apical end. Only 10% in the latter group showed primary regeneration, contrasted with 63–73% in the former groups. It appears that the apico-basal gradient is not changed when pinning is basal or central but that the tendency of the oral end to regenerate apical structures is inhibited by pinning through that end.

C. *Fusion of pieces*

It was possible by the technique described under Methods to fuse portions of animals in various combinations, including (1) apical ends together, (2) the apical end of one against the basal end of the other, (3) basal ends together, and (4) a portion of one animal between portions of two other animals (Fig. 6). The particular combinations tried are outlined in Table II.

TABLE II

Fusion of portions of hydranths

Type of fusion	No.	Age in days after fusion	Results
A. Two pieces			
1. Apical-to-apical			
a. Hypostomes removed; tentacular regions joined	1	13	No primary regeneration. Dedifferentiation of tentacular and gonosomal regions
b. Hypostomes and tentacular regions removed; gonosomes joined	6	13	No primary regeneration. Dedifferentiation of gonosomal region
*c. Stems only	15	7	No primary regeneration
*2. Apical-to-basal (stems only)	9	7	No primary regeneration
*3. Basal-to-basal (stems only)	20	7	No primary regeneration
B. Three pieces Pieces numbered below were fused between basal ends of stalks of 2 complete hydranths			
1. Hypostome	4	10	No primary regeneration. Incorporation into stems
2. Tentacular region	5	10	Extra tentacles in tentacular region; later dedifferentiation
3. Gonosomal region	7	12	No primary regeneration. Dedifferentiation of gonosome
4. Stem	5	19	No primary regeneration, no apparent dedifferentiation

* Pinned through middle of one member.

Several generalizations emerge from an examination of the results recorded in Table II. The first is that primary regeneration of parts excised did not take place when portions of hydranths were fused in apical juxtaposition (Experiment A-1). Primary regeneration could have occurred at the apical end if these parts had been isolated separately. This result illustrates the importance of environment in determining morphogenesis at the cut surfaces of hydranths. A similar general explanation would account for the results of fusing pieces between two other hydranths (Experiments B-1, B-2, B-3 and B-4). Although the grafted piece did undergo slight regeneration in one series (extra tentacles in Experiment B-2), generally it merely underwent dedifferentiation. There was no evidence of an inductive effect of the graft on the differentiation of adjacent regions of the host stems.

A third result is that obtained in the fusions of stems in apical-to-basal or basal-to-basal orientation. In these, either one (Experiment A-2) or two (Experiment A-3) apical ends were freely exposed to the culture medium. One member of the fused pair of stems was pinned through the middle to the substratum as in the simple experiments of isolation. In none of the fused pairs, however, was there any evidence of primary regeneration of tentacles at the free apical end.

Dedifferentiation of tentacular and gonosomal regions in Experiments A-1a and A-1b, B-2 and B-3 appeared to be a response to unfavorable physical and chemical conditions, perhaps comparable to those accounting for dedifferentiation of isolated single hydranths exposed to a hypotonic medium or failing to become attached to the substratum.

Discussion

The experiments reported in this paper have raised several interesting problems. First of all, we should like to understand the differences between what we have called "primary" and "secondary" regeneration. In primary regeneration the apico-basal gradient already established apparently is maintained, and a single new oral end develops within a few days at the apical end of a cut piece. Secondary regeneration depends upon a more profound dedifferentiation of existing structure or upon the outgrowth of hydrorhiza, and therefore takes several days longer than primary regeneration. Several new hydranths may develop in close proximity in secondary regeneration, indicating that the dedifferentiated region has become equipotential. These two types of regenerative response were also encountered by Goldin and Barth (1941) in their experiments on regeneration of expressed coenosarcal fragments of Tubularia as compared with stems retaining the perisarc, and by Brien (1943) in *Clava squamata*. Further investigations, both histological and physiological, are required to reveal how the cells actually behave during both primary and secondary regeneration.

A second problem is to explain why regeneration is favored by attachment to the substratum. Normally, of course, Clava is attached basally and may elongate or contract. An isolated stem continues to exhibit these movements, but it frequently rounds up after a few hours, ceases its motility and after several days degenerates without any sign of regeneration. Possibly apical and basal ends in rounded specimens are brought so closely together that the apico-basal gradient is abolished; perhaps internal pressure inhibits differentiation of new oral structures. Attach-

ment by pinning obviously provides the stem with a practical substitute for its normal attachment, thereby enabling elongation and contraction, conditions which appear to be favorable for primary regeneration. As for secondary regeneration, attachment is a *sine qua non*. Hydrorhiza must have a substratum for continued outgrowth and dedifferentiated pieces need to be attached in order to put out new hydranths.

Two additional problems are raised by the inhibition of primary regeneration in (1) isolated stems pinned through the apical end or (2) stems fused together. In the pinning experiments it may be guessed that the glass rod mechanically changed the environment of the apical cells so that they could not move to the positions usually taken in regeneration or could not divide so readily. Another possible explanation is that the apical end pinned close to the substratum was deprived of oxygen or exposed to accumulated waste products in such concentration that regeneration was inhibited. Experiments on the effect of pinning on the respiratory metabolism of Clava might help resolve this problem. Inhibition by fusion is not unexpected in the experiments of joining two pieces with apical ends together or fusing pieces between two other stems. Cells which, if exposed to the culture medium, would form part of regenerated oral structures would in fusion be joined with neighboring cells and mechanically prevented from organizing into a regenerate. More difficult to explain, however, is the inhibition of primary regeneration in stems united in apical-to-basal or basal-to-basal orientation. The data presented in Table II are too few to give any clear idea of the mechanism of inhibition. The long (12 hours) process of fusion on a glass rod may have altered the stems in some manner inimical to regeneration. Unfortunately, the control experiment of holding a single stem on a glass rod for up to 12 hours before culturing was not tried. It is possible that under the conditions of the experiments there was an oxygen deficiency. The experiments ought to be repeated with larger numbers of animals and with particular attention to environmental conditions known to favor regeneration, namely, pH, temperature, oxygen and removal of waste products.

Summary

1. Observations have been made on regeneration of isolated pieces of hydranths of *Clava leptostyla*, including the hypostome and tentacle-bearing region, the gonosome and the stalk.

2. Stalks isolated in 50% and 80% sea water underwent accelerated dedifferentiation as compared with controls in normal sea water.

3. The distinction is drawn between (1) "primary" regeneration, meaning the differentiation of missing oral or basal ends under the influence of the existing apico-basal gradient and (2) "secondary" regeneration, the delayed development of new hydranths from attached hydrorhiza or dedifferentiated tissue.

4. Primary regeneration at the apical end of isolated stalks usually occurred after anchoring the stem against 2% agar with a fine glass rod thrust through the middle or the basal end. Regeneration was inhibited, however, if the apical end was pinned.

5. Primary regeneration of apical ends was repressed by fusion of pieces of 2 hydranths in apical-to-apical juxtaposition or by fusion of an apical piece between the basal ends of two other hydranths.

6. Apical regeneration likewise failed to occur in fusions of two stalks in apical-to-basal or basal-to-basal orientation.

LITERATURE CITED

BARTH, L. G., 1938a. Oxygen as a controlling factor in the regeneration of Tubularia. *Physiol. Zool.*, **11**: 179–186.

BARTH, L. G., 1938b. Quantitative studies of the factors governing the rate of regeneration in Tubularia. *Biol. Bull.*, **74**: 155–177.

BARTH, L. G., 1940a. The process of regeneration in hydroids. *Biol. Rev.*, **15**: 405–420.

BARTH, L. G., 1940b. The relation between oxygen consumption and rate of regeneration. *Biol. Bull.*, **78**: 366–374.

BARTH, L. G., 1944. The determination of the regenerating hydranth in Tubularia. *Physiol. Zool.*, **17**: 355–366.

BRIEN, P., 1943. Études de la régénération et de la rénovation de l'appareil sexuel chez les Hydroïdes (*Clava squamata* O. F. Müller). *Arch. de Biol.*, **54**: 409–475.

CHILD, C. M., 1929. Physiological dominance and physiological isolation in development and reconstitution. *Arch. f. Entw.*, **117**: 21–66.

GOLDIN, A., AND L. G. BARTH, 1941. Regeneration of coenosarc fragments removed from the stem of *Tubularia crocea*. *Biol. Bull.*, **81**: 177–189.

GOLDIN, A., 1942a. Factors influencing regeneration and polarity determination in *Tubularia crocea*. *Biol. Bull.*, **82**: 243–254.

GOLDIN, A., 1942b. A quantitative study of the interrelationship of oxygen and hydrogen ion concentration in influencing Tubularia regeneration. *Biol. Bull.*, **82**: 340–346.

HARGITT, C. W., 1906. The organization and early development of the egg of *Clava leptostyla* Ag. *Biol. Bull.*, **10**: 207–232.

HARGITT, C. W., 1911. Some problems of coelenterate ontogeny. *J. Morph.*, **22**: 493–549.

MILLER, J. A., 1937. Some effects of oxygen on polarity in *Tubularia crocea*. *Biol. Bull.*, **73**: 369.

MILLER, J. A., 1942. Some effects of covering the perisarc upon tubularian regeneration. *Biol. Bull.*, **83**: 416–427.

MOOG, F., 1941. The influence of temperature on reconstitution in Tubularia. *Biol. Bull.*, **81**: 300–301.

PEEBLES, F., 1931. Some growth-regulating factors in Tubularia. *Physiol. Zool.*, **4**: 1–35.

ROSE, S. M., AND F. C. ROSE, 1941. The role of a cut surface in Tubularia regeneration. *Physiol. Zool.*, **14**: 328–343.

ZWILLING, E., 1939. The effect of the removal of perisarc on regeneration in *Tubularia crocea*. *Biol. Bull.*, **76**: 90–103.

AGGLUTINATION OF FISH AND TURTLE ERYTHROCYTES BY VIRUSES[1]

IGOR TAMM

Hospital of The Rockefeller Institute for Medical Research, New York 21, N. Y.

Following the observation by Hirst (1941) that influenza viruses agglutinate chicken erythrocytes, it was found that several other viruses are capable of agglutinating erythrocytes from certain animal species (Burnet, 1942; Lush, 1943; Levens and Enders, 1945; Nagler, 1942; Burnet and Boake, 1946; Mills and Dochez, 1944; Hallauer, 1949; Lahelle and Horsfall, 1949; Olitsky and Yager, 1949; Sabin and Buescher, 1950). Moreover, it was demonstrated that anti- or immune serum against the corresponding virus inhibits hemagglutination by the agent. As a result, the hemagglutination reaction became a much used technique for measuring either viral or antibody concentration and is useful also in the serologic diagnosis of a number of viral diseases. The reaction of influenza, mumps, and Newcastle disease viruses with erythrocytes proved of special interest when it became evident that these viruses are able to elute spontaneously from erythrocytes with which they combine (Hirst, 1942). As the virus elutes, the erythrocytes lose their capacity to adsorb fresh virus. This loss is accompanied by a reduction in the electrophoretic mobility of the erythrocytes (Hanig, 1948; Ada and Stone, 1950; Stone and Ada, 1950). The eluted virus, however, is capable of combining with and agglutinating fresh erythrocytes (Hirst, 1942). A considerable amount of evidence has accumulated favoring the view that influenza, mumps, and Newcastle disease viruses are capable of effecting an enzymatic alteration of receptor areas on the erythrocyte surface. It appears probable that the receptors are mucoprotein in nature. It should be emphasized that none of these viruses is capable of multiplying in erythrocytes. What role such enzymatic activity has in the series of events leading from virus-host cell combination to multiplication of viruses in and their subsequent release from host cells is not established despite interesting work bearing on this problem (Stone, 1948a; 1948b).

The present study was undertaken in order to extend the range of observations in respect to erythrocyte-virus interaction. Erythrocytes from certain lower classes of vertebrates were employed. In addition to viruses known to cause agglutination of erythrocytes from higher classes of vertebrates, other viruses which have not been reported to agglutinate erythrocytes were studied.

Materials and Methods

Viruses. Ten-day old embryonated chicken eggs were inoculated allantoically with PR8 or FM1 strain of influenza A virus, Lee or MB strain of influenza B virus, swine influenza, or Newcastle disease virus. The eggs were incubated at 35° C. for

[1] Part of the work was carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts.

48 hours, then chilled at 4° C. overnight, and the allantoic fluid harvested. Habel or Enders strain of mumps virus was inoculated in 7-day old chicken embryos and allantoic fluid harvested 5 days later. The allantoic fluids were dialyzed against 80 volumes of 0.1 *M* phosphate buffer at pH 7.1, centrifuged at 3800 g for 10 minutes and the supernatants stored at 4° C. In addition, Lee virus was dialyzed against 0.85 per cent NaCl. Herpes simplex virus, K strain, kindly made available by Dr. E. D. Kilbourne, was inoculated on the chorio-allantoic membrane of 10-day old chick embryos. The membranes were harvested after incubation of the eggs at 35° C. for two days. A 50 per cent suspension was then prepared and homogenized in a modified Waring blendor. The suspension was clarified by centrifugation at 3800 g for 10 minutes and the supernatant stored at 4° C. Semliki Forest virus was inoculated allantoically in 10-day old chick embryos. The eggs were incubated for 30 hours. Allantoic fluid was harvested, dialyzed, centrifuged and stored as described above. The infected embryos, too, were collected and a 50 per cent suspension prepared and stored as described above. Columbia MM strain of mouse encephalomyelitis virus was inoculated intracerebrally in 5-week old white mice. The mice were killed two days later and a 25 per cent suspension of infected brain was prepared as described above. Coxsackie virus (Conn. No. 5 strain) was inoculated in one-day old suckling mice. The mice were killed four days later. A 10 per cent suspension of the carcasses was prepared, homogenized and centrifuged at 3800 g for 30 minutes. The supernatants were stored at 4° C. Pneumonia virus of mice (PVM) was inoculated intranasally in three-week old mice. The mice were killed 6 days later and the lungs collected. A 10 per cent suspension in water was prepared, homogenized, heated at 70° C. for 30 minutes, and clarified by centrifugation at 3800 g for 10 minutes. The supernatant was collected and stored at 4° C. Uninfected control suspensions of each of the various host tissues used were prepared.

In experiments with erythrocytes, untreated viruses or viruses heated at 56° C. for 30 minutes were employed as described below.

Erythrocytes. Blood, obtained with syringe and needle, was mixed with ⅙ volume of a 2.5 per cent solution of sodium citrate. Two or more representatives of each species of animals were bled and their RBC tested. Erythrocytes were washed three times with appropriate diluent (see below) and a 1 per cent suspension prepared in the same diluent. It was found that erythrocytes from dogfish and turtle sedimented more rapidly in the centrifuge than those from mackerel, sea robin or tautog.

Dogfish (*Mustelus canis*). Blood was obtained from the caudal vein. The freezing point depression of blood of marine elasmobranchii is − 1.93 (Smith, 1931) which corresponds to the freezing point depression given by a 3.26 per cent solution of NaCl (Roth and Scheel, 1923). Because of the high concentration of urea in the blood, the diluent chosen for dogfish erythrocytes contained 1.64 per cent NaCl and 2.16 per cent urea (Lutz, 1930). Other salts were omitted.

Mackerel (*Scomber scombrus*), *sea robin* (*Prionotus carolinus*), *tautog* (*Tautogo onitis*). Blood was obtained by cardiac puncture. The freezing point depression of blood of typical marine teleosts is − 0.8 (Smith, 1931). Correspondingly, a 1.37 per cent solution of NaCl was used as the suspending agent (Roth and Scheel, 1923).

Turtle (*Pseudemys elegans*). Blood was obtained by cardiac puncture through a drill hole in the plastron. On the basis of freezing point depressions reported for

various turtles (Botazzi, 1908; Burian, 1910), a 0.86 per cent solution of NaCl was used as the medium for turtle erythrocytes.

Hemagglutination tests. Serial twofold or tenfold dilutions of virus were prepared in 0.4 cc. volumes in the appropriate diluent and 0.4 cc. of 1 per cent erythrocytes added per tube (final concentration 0.5 per cent). The tubes were maintained at 25 or 5° C. for periods of time varying from 10 to 80 minutes, depending on speed of sedimentation and disagglutination of erythrocytes, as indicated below. The tests then were read. The last tube showing definite (2 + to 3 +) agglutination was taken as the end point. Titers are expressed as the reciprocal of the dilution of virus at the end point.

Experimental

Agglutination of fish and turtle erythrocytes by viruses. In the initial experiments with erythrocytes and viruses employed in this study, hemagglutination tests were carried out at 25 and 5° C. with three final concentrations of virus representing

Table I

Agglutination of marine erythrocytes by influenza, mumps and Newcastle disease viruses

Erythrocytes	Virus							
	Influenza A		Influenza B		Swine influenza	Mumps		Newcastle disease
	PR8	FM1	Lee	MB		Enders	Habel	
Dogfish	+*	+	+	+	+	+	+	+
Sea robin	+	+	+	+	+	+	+	+
Mackerel	−†	−	−	+	+	+	+	+
Tautog	+	+	+	+	+	+	+	+
Turtle	−	−	−	+	+	+	+	+

* Plus sign indicates that definite hemagglutination was present at a dilution of 1:20 or higher.
† Minus sign indicates that no evidence of hemagglutination was present at a dilution of 1:2. Because qualitatively identical results were obtained at 25 and 5° C., each sign represents results recorded at both temperatures.

1 : 2, 1 : 20 and 1 : 200 diluted allantoic fluid, mouse lung or mouse brain suspension. Table I summarizes the results of these experiments with various strains of influenza, mumps and Newcastle disease viruses. As can be seen, hemagglutination was observed in the great majority of tests. However, striking specificity was shown with PR8, FM1 and Lee strains of influenza virus which failed to agglutinate mackerel and tautog erythrocytes either at 25 or 5° C.

No agglutination of dogfish, mackerel, sea robin, tautog or turtle erythrocytes was observed under identical conditions with the following viruses: herpes simplex, Semliki Forest, Columbia MM strain of mouse encephalomyelitis, Coxsackie (Conn. No. 5) and pneumonia virus of mice (PVM).

Control experiments with normal allantoic fluid, suspensions of normal mouse lung and mouse brain gave no evidence of agglutination.

Comparison of hemagglutination titers. Lee and MB strains of influenza B virus were employed in comparative experiments with various erythrocytes. It was de-

sired to determine the range of hemagglutination titers at two temperatures: 25 and 5° C. In addition, hemagglutination titers with viruses heated at 56° C. for 30 minutes were measured.

Table II gives the results of representative experiments of this kind. As can be seen, the hemagglutination titers at 25° C. fall within a range of 256 to 4026, with the exception of low titers obtained with Lee virus when sea robin erythrocytes were used and with MB virus and mackerel red blood cells. In the latter instances the titers observed at 60 minutes were lower than those at 30 minutes in contrast to the stable values observed with other virus-erythrocyte combinations within the time interval indicated.

The hemagglutination titers obtained at 5° C. were within the same high range as, or in certain instances somewhat higher than, the titers obtained at 25° C. In

TABLE II

Agglutination of marine erythrocytes by Lee and MB strains of influenza B virus

Treatment of virus	Temp. of agglut.	Lee virus						MB virus				
		Reading	Dog-fish	Mack-erel	Sea robin	Tau-tog	Turtle	Dog-fish	Mack-erel	Sea robin	Tau-tog	Turtle
	° C.											
None	25	1st*	1,024	0	16	1,024	0	2,048	64	256	512	256
		2nd†	1,024	0	4	1,024	0	4,096	8	256	512	256
56° C., 30′	25	1st	1,024	0	64	1,024	0	4,096	128	128	256	128
		2nd	1,024	0	64	1,024	0	4,096	128	128	256	256
None	5	1st	2,048	0	512	1,024	0	4,096	256	512	512	512
		2nd	2,048	0	512	1,024	0	4,096	256	512	512	512

* 1st reading at 20 minutes with dogfish and turtle erythrocytes, and at 30 minutes with mackerel, sea robin and tautog erythrocytes.

† 2nd reading at 40 minutes with dogfish and turtle erythrocytes, and at 60 minutes with mackerel, sea robin and tautog erythrocytes.

particular, Lee virus and sea robin erythrocytes as well as MB virus and mackerel erythrocytes gave high titers at this temperature.

Hemagglutination titers obtained at 25° C. with heated viruses (56° C., 30 minutes) were, in general, closely similar to those observed with unheated agents at this temperature. However, heated Lee virus and sea robin erythrocytes, as well as heated MB virus and mackerel erythrocytes, gave titers somewhat higher than those observed with unheated viruses at this temperature. Moreover, the titers observed were stable, in contrast to those obtained with untreated viruses at this temperature. It should be noted that the titers observed with these two viruses (untreated) at 5° C. were still higher.

Disagglutination of erythrocytes. In view of the evidence that elution of influenza viruses from erythrocytes is accompanied by loss of the ability of such erythrocytes to agglutinate with the same virus (Hirst, 1942), experiments were done to determine whether elution of the virus and disagglutination or dispersion of agglutinated erythrocytes were correlated in time.

The Lee and MB strains of influenza B virus employed were dialyzed against 0.85 per cent NaCl and 0.1 *M* phosphate buffer, respectively. Chicken erythrocytes washed with and suspended in 0.85 per cent NaCl were used. Preliminary experiments were done to determine the most suitable proportions of virus and erythrocytes. If too high relative concentrations of virus are used, the considerable amounts of uncombined virus in the system interfere with measurement of the quantities of virus adsorbed or eluted per unit of time. On the other hand, if too high relative concentrations of erythrocytes are used, the large number of unagglutinated erythrocytes interferes with the counting of disagglutinated cells. These experiments showed that satisfactory adsorption-elution and agglutination-disagglutination curves could be obtained when undiluted allantoic fluid (with a hemagglutination titer of 1024) was mixed with an equal volume of 10 per cent erythrocytes.

Preliminary experiments were also done to determine the optimal temperature for elution, and with Lee virus 14° C. was chosen, whereas with MB virus elution curves were determined at 37° C. This difference indicates that Lee virus has a much faster rate of elution from chicken erythroyctes than MB virus.

The adsorption-agglutination and elution-disagglutination experiments were done as follows: Virus preparation and erythrocyte suspension were mixed at 4° C. in a 25 × 110 mm. test tube with a side arm. Homogeneous distribution of agglutinated and unagglutinated erythrocytes in the mixture was maintained by mechanical stirring at a fixed rate. At intervals, aliquots were removed with a pipette via the side arm. The erythrocytes in a part of each aliquot were promptly sedimented by centrifugation. The supernatant was collected and the concentration of virus determined by means of hemagglutination titration. The other portion of the aliquot was diluted 1 : 5 with 0.85 per cent NaCl and unagglutinated erythrocytes were counted in a hemocytometer. After a suitable period of time, the reaction mixture was brought to the temperature at which elution-disagglutination could be followed optimally and additional aliquots were removed and examined as described above.

Figure 1 gives the results of representative experiments with Lee and MB viruses. As can be seen, elution of virus and disagglutination of erythrocytes followed essentially parallel courses.

In experiments with erythrocytes from marine species, a large number of instances of spontaneous disagglutination of erythrocytes was observed. The results with Lee virus and sea robin erythrocytes and with MB virus and mackerel erythrocytes were discussed above. In addition, similar evidence was obtained with dogfish, tautog and turtle erythrocytes. These experiments were carried out at 25° C. The period of observation was prolonged to four hours by re-suspension of sedimented erythrocytes by shaking at regular intervals. Disagglutination of erythrocytes from each of the five species employed was observed with two or more influenza virus strains and with Newcastle disease virus. Agglutination of erythrocytes by mumps virus was followed by spontaneous disagglutination in the case of turtle, mackerel and dogfish erythrocytes.

In different virus-erythrocyte systems, loss of agglutinability appeared after strikingly different periods of time. The rate of action of Newcastle disease and Lee viruses on dogfish and sea robin erythrocytes was found to be considerably faster than that of other viruses employed, including PR8, FM1, MB, swine and mumps. Moreover, marked differences were noted in respect to the time required by different erythrocytes to lose their agglutinability when mixed with one and the same virus,

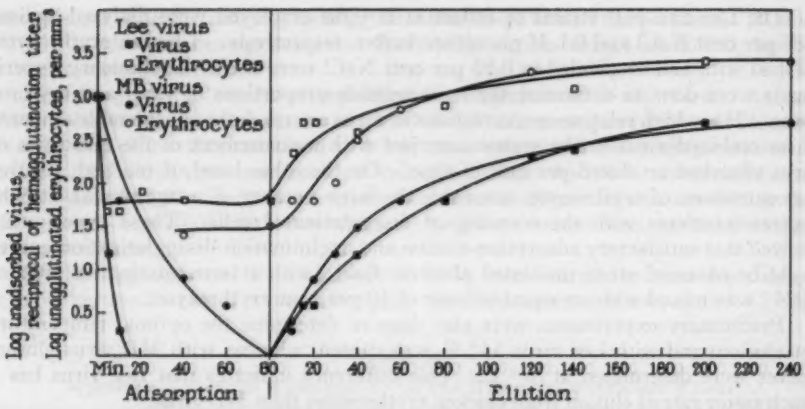


FIGURE 1. Relation of adsorption and elution of virus to agglutination and disagglutination of erythrocytes. With both Lee and MB viruses, adsorption was carried out at 4° C. Elution was permitted to take place at 14° C. with Lee virus, and at 37° C. with MB virus. In both experiments the control virus titer was 1024. Chicken erythrocytes, final concentration 5 per cent, were used.

e.g., employing MB virus, longer periods were required with tautog, sea robin and dogfish erythrocytes than with mackerel or turtle cells. In no case was evidence of disagglutination observed when tests were done at 4° C.

It appeared, therefore, that disagglutination was a regular phenomenon and that the exceptional instances where disagglutination was not observed may have been due to experimental conditions used: if temperatures higher than 25° C. and periods longer than 4 hours had been employed, disagglutination might have occurred in such instances.

DISCUSSION

The finding that influenza, mumps and Newcastle disease viruses are capable of agglutinating erythrocytes from teleosts and elasmobranchii is of interest because it focuses attention on the extremely broad species range of this reaction: the agglutination of reptilian, amphibian, avian and mammalian erythrocytes by representatives of this group of viruses has been demonstrated previously (Smadel, 1948). Studies with chicken erythrocytes have yielded suggestive evidence that in the hemagglutination reaction influenza, mumps and Newcastle disease viruses combine with receptor groups on erythrocytes which are mucoprotein in nature (de Burgh *et al.*, 1948; Hirst, 1948). Strong, although indirect, support for this contention has come from studies with mucoid components of tissues and body fluids which react with influenza, mumps and Newcastle disease viruses (Tamm and Horsfall, 1952). One of these components, a mucoprotein present in normal human urine, has been shown, following isolation and purification, to be a single homogeneous substance by electrophoretic and ultracentrifugal criteria (Perlmann *et al.*, 1952; Bugher, 1951). The features which characterize virus-erythrocyte interaction also characterize that between mucoprotein from urine and influenza, mumps or Newcastle disease viruses:

combination between the reactants is followed by emergence of free virus and demonstrable alteration in the mucoprotein. The altered mucoprotein is not able to combine with virus and its electrophoretic mobility is reduced (Perlmann *et al.*, 1952). As in the case of virus-erythrocyte interaction, the rate at which the virus causes alteration of the mucoprotein is considerably faster with certain viruses than with others. For example, the rate of Lee or Newcastle disease virus action on the mucoprotein is more rapid than that of MB, FM1, mumps or swine influenza viruses (Tamm and Horsfall, 1952). The findings reported in this study are in agreement with these observations.

Thus, it appears probable that erythrocytes from species ranging from man to the dogfish possess similar surface receptor areas with which influenza, mumps or Newcastle disease viruses are able to combine, and which they alter during the process of elution. However, within the species range of virus-erythrocyte interaction, instances can be found in which agglutination does not take place, as illustrated by the failure of PR8, FM1 or Lee viruses to agglutinate mackerel or turtle erythrocytes. Structural fit as well as adsorptive forces may be factors involved in this phenomenon for which at present an explanation is lacking.

In contrast to the group of viruses discussed above, pneumonia virus of mice, the various mouse encephalomyelitis viruses, and Japanese B encephalitis virus are agents which agglutinate erythrocytes from only a very few animal species. Agglutination of erythrocytes by these viruses takes place under conditions which are different in each case. These agents do not show spontaneous elution and nothing definite is known about the chemical nature of the groupings on the red cell surface with which these viruses combine. It should be emphasized that numerous viruses have failed to give the hemagglutination reaction with any erythrocytes against which they were tested.

Summary

1. Dogfish, sea robin or tautog erythrocytes are agglutinable by all strains of influenza, mumps or Newcastle disease viruses employed.

2. Mackerel and turtle erythrocytes are agglutinable by mumps or Newcastle disease viruses as well as by some strains of influenza B virus. In the presence of these agents, agglutinable erythrocytes lose their capacity to be agglutinated by the same virus and spontaneous elution of the agent occurs. Elution of virus from erythrocytes and disagglutination appear to occur concurrently.

3. Among a number of other viruses tested, none was capable of agglutinating erythrocytes employed in this study.

LITERATURE CITED

Ada, G. L., and J. D. Stone, 1950. Electrophoretic studies of virus-red cell interaction: mobility gradient of cells treated with viruses of the influenza group and the receptor-destroying enzyme of *V. cholerae*. *Brit. J. Exp. Path.*, **31**: 263–274.

Bottazzi, F., 1908. Osmotischer Druck und elektrische Leitfähigkeit der Flüssigkeiten der einzelligen, pflanzlichen und tierischen Organismen. *Ergeb. Physiol.*, **7**: 161–402.

Bugher, J. C., 1951. Personal communication.

Burian, R., 1910. Funktion der Nierenglomeruli und Ultrafiltration. *Pflüger's Archiv. ges. Physiol.*, **136**: 741–760.

Burnet, F. M., 1942. The affinity of Newcastle disease virus to the influenza virus group. *Australian J. Exp. Biol. and Med. Sc.*, **20**: 81–88.

BURNET, F. M., AND W. C. BOAKE, 1946. The relationship between the virus of infectious ectromelia of mice and vaccinia virus. *J. Immunol.*, **53**: 1–13.

DE BURGH, P. M., P.-C. YU, C. HOWE AND M. BOVARNICK, 1948. Preparation from human red cells of a substance inhibiting virus hemagglutination. *J. Exp. Med.*, **87**: 1–9.

HALLAUER, C., 1949. Agglutination von Hammelerythrocyten durch murine Poliomyelitis-virusstämme. 4th International Congress of Microbiology, July 20–26, 1947, Copenhagen. Report of Proceedings, p. 257.

HANIG, M., 1948. Electrokinetic change in human erythrocytes during adsorption and elution of PR8 influenza virus. *Proc. Soc. Exp. Biol. and Med.*, **68**: 385–392.

HIRST, G. K., 1941. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science*, **94**: 22–23.

HIRST, G. K., 1942. Adsorption of influenza hemagglutinins and virus by red blood cells. *J. Exp. Med.*, **76**: 195–209.

HIRST, G. K., 1948. The nature of the virus receptors of red cells. I. Evidence on the chemical nature of the virus receptors of red cells and of the existence of a closely analogous substance in normal serum. *J. Exp. Med.*, **87**: 301–314.

LAHELLE, O., AND F. L. HORSFALL, JR., 1949. Hemagglutination with the GDVII strain of mouse encephalomyelitis virus. *Proc. Soc. Exp. Biol. and Med.*, **71**: 713–718.

LANNI, F., D. G. SHARP, E. A. ECKERT, E. S. DILLON, D. BEARD AND J. W. BEARD, 1949. The egg white inhibitor of influenza virus hemagglutination. I. Preparation and properties of semipurified inhibitor. *J. Biol. Chem.*, **179**: 1275–1287.

LEVENS, J. H., AND J. F. ENDERS, 1945. The hemoagglutinative properties of amniotic fluid from embryonated eggs infected with mumps virus. *Science*, **102**: 117–120.

LUSH, D., 1943. The chick red cell agglutination test with the viruses of Newcastle disease and fowl plague. *J. Comp. Path. and Therap.*, **53**: 157–160.

LUTZ, B. R., 1930. The effect of adrenalin on the auricle of elasmobranch fishes. *Amer. J. Physiol.*, **94**: 135–139.

MILLS, K. C., AND A. R. DOCHEZ, 1944. Specific agglutination of murine erythrocytes by a pneumonitis virus in mice. *Proc. Soc. Exp. Biol. and Med.*, **57**: 140–143.

NAGLER, F. P. O., 1942. Application of Hirst's phenomenon to the titration of vaccinia virus and vaccinia immune serum. *Med. J. Australia*, **1**: 281–283.

QLITSKY, P. K., AND R. H. YAGER, 1949. Hemagglutination by Columbia SK, Columbia MM, mengo encephalomyelitis and encephalomyocarditis viruses: experiments with other viruses. *Proc. Soc. Exp. Biol. and Med.*, **71**: 719–724.

PERLMANN, G. E., I. TAMM AND F. L. HORSFALL, JR., 1952. An electrophoretic examination of a urinary mucoprotein which reacts with various viruses. *J. Exp. Med.*, **95**: 99–104.

ROTH, W. A., AND K. SCHELL, 1923. Landolt-Börnstein physikalisch-chemische Tabellen. Berlin, Springer, ed. 5, **2**: 1452.

SABIN, A. B., AND E. L. BUESCHER, 1950. Unique physico-chemical properties of Japanese B encephalitis virus hemagglutinin. *Proc. Soc. Exp. Biol. and Med.*, **74**: 222–230.

SMADEL, J. E., 1948. Serologic reactions in viral and rickettsial infections. Viral and rickettsial infections of man, edited by Thomas M. Rivers, Philadelphia, Lippincott, pp. 67–96.

SMITH, H. W., 1931. The absorption and excretion of water and salts by the elasmobranch fishes. II. Marine elasmobranchs. *Am. J. Physiol.*, **98**: 296–310.

STONE, J. D., 1948a. Prevention of virus infection with enzyme of *V. cholerae*. I. Studies with viruses of mumps-influenza group in chick embryos. *Australian J. Exp. Biol. and Med. Sc.*, **26**: 49–64.

STONE, J. D., 1948b. Prevention of virus infection with enzyme of *V. cholerae*. II. Studies with influenza virus in mice. *Australian J. Exp. Biol. and Med. Sc.*, **26**: 287–298.

STONE, J. D., AND G. L. ADA, 1950. Electrophoretic studies of virus-red cell interaction: additive effect of viruses of the influenza group and the receptor-destroying enzyme of *V. cholerae*. *Brit. J. Exp. Path.*, **31**: 275–284.

TAMM, I., AND F. L. HORSFALL, JR., 1952. A mucoprotein derived from urine which reacts with influenza, mumps and Newcastle disease viruses. *J. Exp. Med.*, **95**: 71–97.

RESPIRATORY STUDIES OF SINGLE CELLS. I. METHODS[1]

P. F. SCHOLANDER, C. L. CLAFF AND S. L. SVEINSSON

Department of Biological Chemistry and Laboratory for Surgical Research, Harvard University Medical School, Boston, Mass.

For a detailed study of respiratory events in single cells, even large ones such as the commonly used marine eggs or protozoans, a stability of a few $\mu\mu$l/hour[2] or better and a sensitivity down to a fraction of a $\mu\mu$l are desirable. Only one method (Zeuthen, 1943) has been described so far that approaches this order of magnitude. By modifying and reducing drastically the conventional Cartesian diver respirometer (Linderstrøm-Lang, 1937, 1942, 1943; Linderstrøm-Lang and Glick, 1938; Boell, Needham and Rogers, 1939; Holter, 1943), he was able to achieve a stability of 0–20 $\mu\mu$l/hour and a sensitivity of about 20 $\mu\mu$l. With this 0.1 μl diver he measured the oxygen consumption of a single polychaete worm egg and of single amoebae. In another series the method was used in a study of the single, half, whole and syncytial egg of an ascidian (Andresen, Holter and Zeuthen, 1944; Holter and Zeuthen, 1944).

Large single amphibian eggs have been studied by means of larger and less sensitive modifications of the conventional Cartesian diver (Zeuthen, 1946).

In this paper we present a method for micro respiratory studies of single cells that so far has yielded results with a sensitivity of about 0.2 $\mu\mu$l and a stability (drift) from 0–10 $\mu\mu$l per hour. The method is in some respects simpler than the 0.1 μl Cartesian diver and seems to offer possibilities for further refinements. It is to be hoped it will prove its usefulness among flotation methods for measuring respiration, the development of which has been so successfully carried out at the Carlsberg Laboratory.

I. The Respirometer

In 1911 Krogh developed a method for micro gas analysis which he named microscopical gas analysis and which consisted essentially of measuring the diameter of gas bubbles under the microscope before and after absorption of the CO_2 and O_2. In 1941, it seemed to one of the authors that it might be possible to place a cell in a small chamber full of water together with a bubble of oxygen. The chamber would be in pressure equilibration with the outside water bath by means of a fine capillary connecting space. The cell would consume the bubble and the rate of shrinkage could be measured through the microscope. The stability of a bubble in such a system without a cell was tested, using a chamber identical with the ones used at present, and was found very good. However, as the bubble shrinks from the oxygen consumption the surface tension builds up an increasing gas pressure in it, according to the relation $P = \frac{4T}{D}$, where T = the

[1] This work was supported by a grant from the William F. Milton Fund.

[2] One $\mu\mu$l is defined as one millionth of one cubic millimeter = liter $\times 10^{-12}$.

surface tension and D the diameter. If T is taken as 73 dynes/cm., the pressure in the bubble due to the surface tension will be $\frac{2200}{D}$ mm. Hg when D is measured in microns. Hence, a 10 μ bubble has an overpressure of 220 mm. Hg, a 100 μ bubble an overpressure of 22 mm. Hg, etc. It is therefore clear that this principle can only be applied on a manometric basis where the pressure is so adjusted that the bubble volume is kept constant. As in any manometric method, the change in pressure will reflect the oxygen consumption when the volume of the gas and liquid content of the chamber are known. The problem therefore became how to know most accurately that the bubble was being maintained at constant volume. By microscopic measurement of the bubble the volume could be estimated with an accuracy of no more than three per cent, which was not very satisfactory. A much more sensitive way of ascertaining the volume constancy of our bubble would be to make use of the extreme accuracy whereby the density of minute objects can be determined by flotation (Linderstrøm-Lang and Lanz, 1938). The problem would then be to stick the bubble in the respiration chamber onto a hydrophobic minute weight and then to regulate the pressure so that the bubble would balance the weight suspended in the water.

In the Cartesian diver the constancy of the gas volume of the chamber is ascertained by floating the whole chamber. In the present system a minute reference diver is floated in the respiratory chamber containing the cell. The gas phase is less than one per cent of the water phase, and the pressure effect by respiratory CO_2 can, at most, amount to about three per cent of the changes in the oxygen tension, even in acid media. This is due to the much higher solubility of CO_2 than oxygen. At slight alkalinity the CO_2 pressure will remain practically zero. Usually, therefore, no special provision for CO_2 absorption need be made.

A. Principle of the method

A minute respiration chamber is filled with a medium which communicates to the same medium on the outside through a fine capillary space. This space serves as a diffusion barrier, but permits free pressure transmission to the chamber from the outside medium, upon which controlled pressure can be applied. The respiration chamber contains, besides the cell, a little bubble adhering to a minute hydrophobic weight. The pressure is adjusted so that the bubble always just suspends the weight to a constant height in the respiratory chamber. We have then a constant volume system, where the pressure changes, read on the manometer, multiplied by a factor calculated from known constants give the oxygen consumption. The CO_2 is effectively held in solution by the large liquid to gas ratio of the system and amounts to three per cent or less of the factor.

B. Construction (Fig. 1)

The apparatus is simple and can be assembled in almost any laboratory from conventional equipment. It consists of the respiratory chamber assembly with the reference diver, accessories for making the reference diver and for charging the respirometer, the manometer with pressure control, the water bath, and optical equipment.

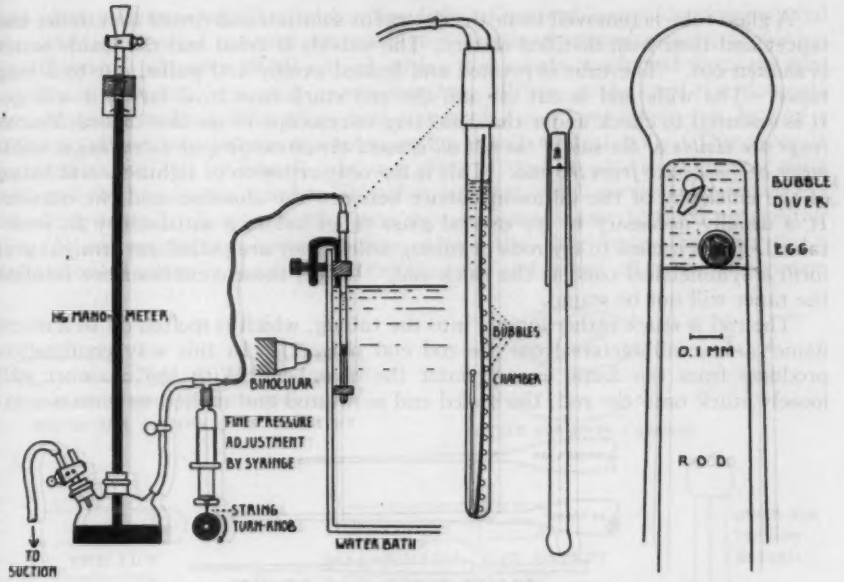


FIGURE 1. Respiration measurement of single cell by means of reference diver.

1. Respiratory chamber assembly

This consists of the chamber, the chamber tube, and the plastic reference diver.

a. Preparation of glass tubing. The respiration chamber is made from gently heated, soft glass.[3] We have not been able to use cleaning solution or detergents in the finished chambers as this would often produce drastic toxic effects on Paramecium, even after lengthy boiling and rinsing in water. All tubing is therefore cleaned before making the chamber. The roundest possible (check by micrometer) soft glass tubing is selected (2.5–3 mm. OD), and is cut into 50 or so pieces 10 cm. long. They are then put in a beaker with 2% Dreft (or other detergent), boiled for a few minutes and left covered by the same solution for future use.

b. Making the respiration chamber (Fig. 2). A soft glass rod, 2–3 mm. thick and as round as possible, is heated evenly by rotation in a flame and pulled out straight to form a symmetrical cone with a long taper, reaching the desired diameter of about 0.3 mm. while still tapered. The desired diameter is determined by a micrometer and the rod cut off at that point, with the least possible chipping. We have found a triangular India honestone to be a better glass cutter for these dimensions than a sharp Carboloy steel edge (which cuts the finger better than the glass). The thick end is cut off and flamed to a knob.

[3] It was found that in the dimensions here used, a freshly made respiration chamber of Pyrex glass is highly toxic to some cells, Paramecium, for instance.

A glass tube is removed from the detergent solution and rinsed first under the faucet and then with distilled water. The outside is dried and the inside water is shaken out. The tube is rotated and heated evenly and pulled out to a long taper. The wide end is cut off and the rod stuck into it as far as it will go. It is essential to check under the dissecting microscope to see *that colored Newton rings are visible at the end of the rod all around the circumference extending a millimeter or more back from the end.* This is the only criterion of tightness and hence of the efficiency of the diffusion barrier between the chamber and the outside. It is usually necessary to try several glass tubes before a satisfactory fit is obtained. It is useless to try rods or tubing unless they are pulled out straight and form a symmetrical cone at the thick end. Unless these conditions are fulfilled the taper will not be round.

The rod is stuck rather loosely into the tubing, which is melted off in a micro flame[4] a few millimeters from the rod end (Fig. 2). In this way combustion products from the flame cannot enter the chamber. With the chamber still loosely stuck onto the rod, the sealed end is rotated and melted to form a sym-

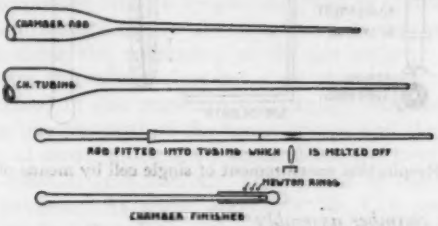


FIGURE 2. Making the respiration chamber.

metrical knob. The rod is pressed in carefully and the chamber is measured. The length should be 1–1.5 times the diameter. If it is too long loosen the rod and heat the knob again, until the desired length of chamber is obtained. The sleeve of the chamber is cut off at a total length of about 5–6 mm. The chamber is now ready for use.

c. The chamber tube (Fig. 1). The respiratory chamber rests during the experiment in a chamber tube about 0.5 × 12 cm., which is fastened to a clamp by means of a section of rubber tubing, so that it can be moved in all directions. The clamp screws onto the front of the water bath. A fine polyethylene tubing, 1.5–2 mm. OD, connects the tube with the pressure system. The tube also holds a tension equilibrating device, consisting of a piece of Saran filament, 1.5–2 mm. thick, pitted every 3 mm. with a sharp needle point. When the pressure is lowered to generate the bubble on the diver, bubbles will also form at each pit and this will facilitate the establishment of the same gas tension in the water outside and inside the respiration chamber.

d. The reference diver (Fig. 3). A piece of Saran tubing,[5] 10 cm. long and about 5 mm. OD, is rotated and very gently heated 10 cm. or so above a low Bunsen flame. When carefully done the tubing will turn clear and melt together

[4] S. S. White (Philadelphia, Pa.) Orthodontic Burner No. 6.
[5] Manufactured by the Dow Chemical Co., Midland, Michigan.

and form a thinner capillary with a fine hole. One end is quickly clamped and the other end pulled out and stretched to a length of several meters, forming a fine filament. A piece 50–60 μ thick with a 1–2 μ hole is selected from the filament. It is wound on a card for safe keeping. While on the card the filaments are measured under a microscope and a piece 2–3 cm. long is cut out, having a uniform, accurately known diameter and a small central hole. The piece is fastened at one end by Scotch tape to a piece of a white plastic millimeter ruler attached to the top of a number 10 rubber stopper, which serves as a convenient "working table." A splinter of a new xylene-cleaned razor blade is twisted off (Fig. 3) and serves as a knife. Before cutting the diver under the binocular a little distilled water is run down on the filament and wiped off with clean gauze.

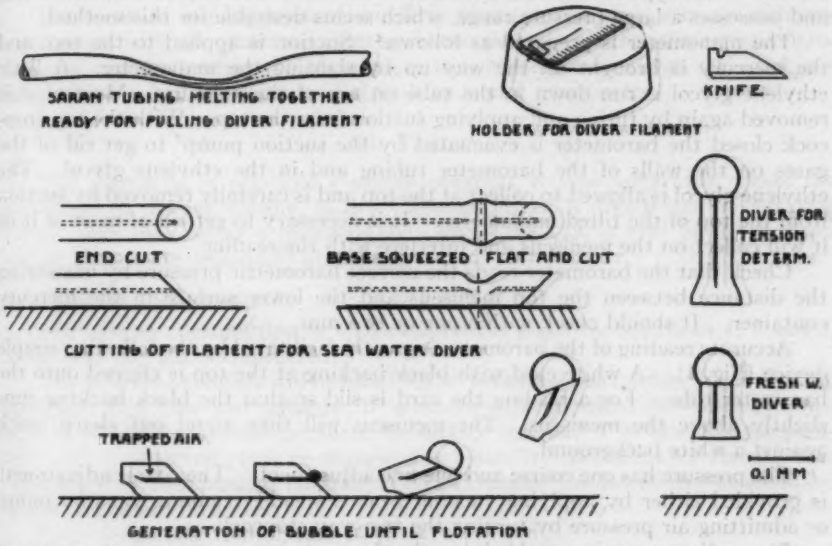


FIGURE 3. Preparing reference diver and bringing it to flotation.

We have used two types of reference divers. One has the top cut obliquely to prevent the bubble from hitting the chamber wall, which is hydrophobic in sea water. The other type has a straight cut top and can usually be used in fresh water, which wets the chamber walls.

To cut the oblique diver, the holder is placed under the binocular and the filament held down with a washed and dry finger. The end is cut with the very tip of the razor blade, slanting it at a 45° angle against the table. The diver filament must be seen to have air in the central hole where it is cut. The cut must be clean and sharp, leaving the hole open. Roll the filament over 180° and squeeze the base of the diver flat with the back side of the knife point, so that it is seen that the capillary closes. Then cut straight. The minute diver usually adheres to the knife point and is transferred directly into the already loaded respiration chamber. It can also be stored for some time in a drop of

water without losing the air that is trapped in the capillary. It can then be transferred somewhat more readily from the drop by means of a braking pipette (Holter, 1943; Claff, 1947) or micrometer burette provided with flexible plastic micro tubing ending in a fine glass tip.

A straight cut diver for fresh water or tension work is basically made the same way.

2. Manometer and pressure control (Fig. 1)

For the pressure measurements we have employed a closed mercury manometer of the same type as used in the Van Slyke-Neill manometric blood gas apparatus. With oxygen-charged water it gives a useful scale of about 600 millimeters. This type of manometer obviates the need for thermobarometer control and possesses a large pressure range, which seems desirable for this method.

The manometer is prepared as follows.[6] Suction is applied to the top, and the mercury is brought all the way up by slanting the manometer. A little ethylene glycol is run down in the tube on top of the mercury. Most of it is removed again by tilting and applying suction from the top. With the top stopcock closed the barometer is evacuated by the suction pump[7] to get rid of the gases on the walls of the barometer tubing and in the ethylene glycol. The ethylene glycol is allowed to collect at the top and is carefully removed by suction from the top of the tilted manometer. It is necessary to get rid of most of it or it will collect on the meniscus and interfere with the reading.

Check that the barometer reads the correct barometric pressure by measuring the distance between the top meniscus and the lower surface in the mercury container. It should check within one or two mm.

Accurate reading of the barometer is greatly facilitated by the following simple device (Fig. 1). A white card with black backing at the top is clipped onto the barometer tube. For a reading the card is slid so that the black backing runs slightly above the meniscus. The meniscus will then stand out sharp black against a white background.

The pressure has one coarse and one fine adjustment. The coarse adjustment is provided either by applying vacuum from a steadily running vacuum pump or admitting air pressure by turning the two-way stopcock.

Fine adjustment is provided by the 5 cc. syringe arrangement shown in Figure 1. The syringe is kept lubricated by applying heavy oil around the barrel seal. The plunger must move smoothly without sticking.

3. Water bath

An ordinary large (40 liter) glass aquarium tank is used as water bath. It is insulated by Celotex or several layers of corrugated paper, except for the upper six or seven centimeters front and back. In front of the bath rests a horizontal binocular, preferably with rack and pinion up and down, and in back of the bath opposite the microscope a small fluorescent lamp. The water bath must be kept within $\pm 0.01°$ C. or better of the desired temperature. If an automatic thermoregulator is used, it is imperative to check the magnitude of the cycling on a Beckmann thermometer during an hour's time to be sure that it does not exceed

[6] For more detailed description see Peters and Van Slyke, 1932, p. 273.

[7] A laboratory suction-blower unit will do.

0.01 degree. The heating and cooling sources should be as gentle as possible. An infrared lamp suspended above the water bath and connected with a rheostat makes an excellent source of heat, and a mere trickle of cool water on the stirring propeller may serve as a cold source. By regulating the rheostat the temperature can easily be controlled within the desired limits, even by hand.

4. Optical equipment

For observing the cell and the reference diver, any type of binocular dissecting microscope mounted horizontally can be used. It must be provided with an ocular micrometer scale which can be calibrated against the grid of a blood counting chamber or even against a millimeter ruler stuck into the water bath. From 40 up to 100 times magnification has been found satisfactory.

For accurate measurement of the diver filament, egg sizes, etc., an ordinary microscope with an ocular micrometer is necessary. Another dissecting microscope is used for loading the chamber, cutting the reference diver, etc., and it is convenient to have it also provided with an ocular micrometer.

II. Theory

The system is in principle a simple constant volume apparatus, where the constancy of the gas volume, as in a Cartesian diver, is determined by flotation rather than by reading a meniscus, but where the CO_2 is held in the medium rather than being separately absorbed.

A. Equations and constants

The oxygen consumption or CO_2 evolution is given by equations 1, 2 and 3, when only one gas is considered.

The following symbols will be used: V_B = volume of gas bubble; V_L = volume of liquid; P = observed pressure; W = water vapor tension; N = nitrogen tension; S = bubble pressure produced by surface tension;[8] T = absolute temperature; ΔP = observed pressure change; α = solubility coefficient for the gas that changes; F = the chamber constant. STPD means "measured dry at standard temperature and pressure."

Gas volume (STPD) at start is

$$V_B \frac{273}{T}\left(\frac{P-(W+N-S)}{760}\right)+V_L\alpha\left(\frac{P-(W+N-S)}{760}\right)=A. \quad (1)$$

Gas volume (STPD) at end is

$$V_B \frac{273}{T}\left(\frac{P-(W+N-S)-\Delta P}{760}\right) + V_L\alpha\left(\frac{P-(W+N-S)-\Delta P}{760}\right)=B. \quad (2)$$

$$\text{Gas consumed (or produced)} = A-B=\Delta P\left(\frac{V_B\frac{273}{T}+V_L\alpha}{760}\right), \quad (3)$$

[8] See page 157.

which is the well-known formula for calculating the flask constant in a Warburg manometric apparatus.

In a system where CO_2 is not separately absorbed, as in our system, an expression for the oxygen consumption ΔO_2 in terms of the pressure reading ΔP is given by the following equations:

$$\Delta V_{O_2} = \Delta P_{O_2}\left(\frac{V_B\frac{273}{T} + V_L\alpha}{760}\right), \tag{4}$$

$$\Delta V_{CO_2} = \Delta P_{CO_2}\left(\frac{V_B\frac{273}{T} + V_L\alpha}{760}\right). \tag{5}$$

Assuming $RQ = 1$,

$$\Delta V_{O_2} = \Delta V_{CO_2}, \tag{6}$$

$$\Delta P = \Delta P_{O_2} - \Delta P_{CO_2}. \tag{7}$$

Solved for ΔV_{O_2} the final equation gives the oxygen consumption:

$$\Delta V_{O_2} = \Delta P\left(\frac{1}{\frac{760}{V_B\frac{273}{T} + V_L\alpha_{O_2}} - \frac{760}{V_B\frac{273}{T} + V_L\alpha_{CO_2}}}\right) = \Delta P \times F. \tag{8}$$

It will be noted that pressures which remain constant, such as those contributed by water vapor, nitrogen and surface tension of the bubble, cancel out in equations 1 and 2 and are of no influence as long as they remain constant. (See derivation of equation 3 in Dixon, 1943.)

Besides the pressure difference ΔP read on the manometer, we must know T, V_B, V_L, α_{O_2}, and α_{CO_2}. T is equal to the water bath temperature. V_B is very small and can be estimated closely enough from its measurement in the ocular eyepiece. It can be better estimated by knowing the volume per millimeter of the diver filament and calculating the volume of the diver from its measured length. With a specific gravity of Saran of 1.7, the bubble volume will be the diver volume times 0.7.

V_L is obtained from measuring in the binocular the diameter and the height of the chamber, allowing for the rounded upper corners. In order to save repeated calculations a chart is prepared, where on a log log paper the diameter from 0.1–1 mm. is plotted against the volume per millimeter height, which at 0.2 mm. diameter is 314,000 $\mu\mu$l and at 0.8 is 503,000 $\mu\mu$l. A straight line is drawn through these points. The volume/mm. read on the graph multiplied by the height in millimeters gives the chamber volume in $\mu\mu$l. The cell may conveniently and without significant error be considered water. The diver volume plus the bubble volume is usually smaller than the error in the estimation of the chamber volume and can then be ignored. Usually, therefore, V_L can be taken equal to the chamber volume.

The absorption coefficient of oxygen, α_{O_2}, for fresh water may be found in any handbook, and for sea water of different salinities and temperatures it may be found by interpolation of data given in Sverdrup, Johnson and Fleming (1942).

The absorption coefficient at different temperatures for carbon dioxide (α_{CO_2}) in acid media can be found in standard handbooks and for acid sea water in the above mentioned book. In neutral or alkaline media the CO_2 retention can without much difficulty be determined, as described below.

B. The pressure effect of CO_2 production

From equations 1 and 2 it can easily be shown that the ratio $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}}$ depends upon the ratio of the solubility coefficients and the ratio of the liquid volume to the gas volume. In an all gas phase the carbon dioxide will of course exercise its full pressure, so that $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}} = 1$, whereas in an all liquid phase the ratio becomes $\frac{\Delta P_{O_2}}{\Delta P_{CO_2}} = \frac{\alpha_{CO_2}}{\alpha_{O_2}}$. From Figure 4 it will be seen that when the gas volume gets smaller than 1% of the liquid volume, the pressure effect of the CO_2 gets to be very near to the lowest possible, namely $P_{O_2} \times \frac{\alpha_{O_2}}{\alpha_{CO_2}}$. In a system like this one we must therefore strive to keep the gas volume less than 1% of the liquid volume. In most of our work it has been 0.5% or less.

With an increasing alkalinity the pressure effect of CO_2 will naturally get smaller and smaller. Exactly what it will be can be determined in the following way.

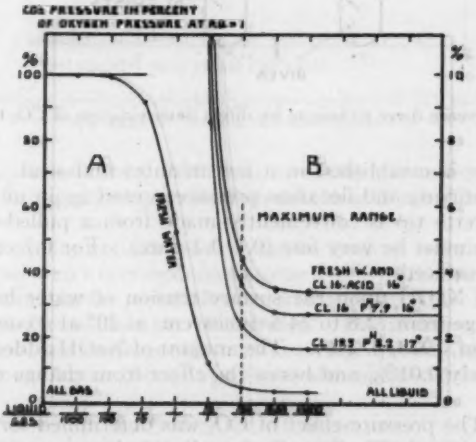


FIGURE 4. Pressure effect of carbon dioxide production in per cent of the pressure effect of an equal oxygen consumption when the gas-to-liquid ratio in the respirometer is varied. A. The entire curve for fresh water with ordinate units to the left. B. The lower part of three curves magnified ten times, with ordinate units to the right.

1. Determination of the CO_2 pressure

a. Principle. Increasing, known amounts of CO_2 gas are added anaerobically to a series of syringes containing 10 cc. sea water each. The CO_2 is dissolved quantitatively by chilling the syringes. The charged water is transferred with negligible gas loss to a long-necked 1 cc. flask in an accurately regulated water bath (Fig. 5). A micro reference diver is placed in the flask which is connected to a manometer and pressure regulating device (the same as for the respirometer chamber). The diver is brought to flotation and the pressure is read. This represents the total gas pressure plus the surface tension effect. A few cubic millimeters of strong NaOH are added from a microburette to make the sea water alkaline. The reference diver is again brought to flotation. The difference in pressure gives the CO_2 effect directly in mm. Hg.

b. Details of procedure. The flask is provided with a spiral depression so it can be vigorously stirred by twisting back and forth while still connected with the manometer. The diver is long so that the bubble will completely occupy the upper surface and hence will assume the same radius after collapse.

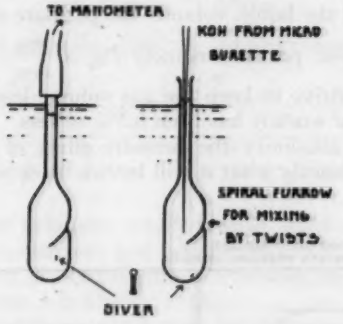


FIGURE 5. Reference diver technique for direct determination of CO_2 tension in liquid.

Flotation pressure is established in a few minutes and read. The chamber is twisted to effect stirring and flotation pressure is read again until it attains constancy. The burette tip is conveniently made from a pulled-out polyethylene micro tubing. It must be very fine (0.1–0.2 mm.). For 0.7 cc. sea water, 1.50 mm.[3] 1 *N* NaOH sufficed.

The action of NaOH upon the surface tension of water has been found to amount to a change from 72.8 to 74.6 dynes/cm. at 20° at a concentration of 5% (Landolt-Börnstein, 1923, p. 240). The amount of NaOH added to the sea water gives approximately 0.01%, and hence the effect from change of surface tension is negligible.

c. Results. The pressure effect of CO_2 was determined for the different sea waters which were used (Fig. 6). Carbon dioxide has greater pressure effect in acid sea water than in fresh water, because the salinity lowers the CO_2 solubility. The pressure effect decreases rapidly with increasing pH. At pH 8.2–8.4 it is only half or less that of fresh water. From the curves the average retention of

the CO_2 can be calculated and hence the pressure effect of CO_2 for different gas-to-liquid ratios at different pH and salinity can be estimated (Fig. 4).

It will be seen that in all our experiments with sea water in the chamber, the pressure effect of the CO_2 has only amounted to 2–3% of the oxygen pressure effect. This correction is included in equation 8, but can usually be ignored because the chamber volume, and certainly the volume or weight of the respiring material, are known with less accuracy.

It is, however, important to know the magnitude of the CO_2 effect for the interpretation of any irregularities or bumps on the curves, and it is then well to realize that a maximum metabolic change in *RQ* from 0.7 to 1 will only show up as a 1% or less error in the oxygen consumption. Similarly, acid formation must be at least equivalent to the oxygen consumption on a molar basis before it begins to show up. In other words, the CO_2 binding properties of straight unbuffered sea water, such as used in our experiments, hold the CO_2 pressure effect

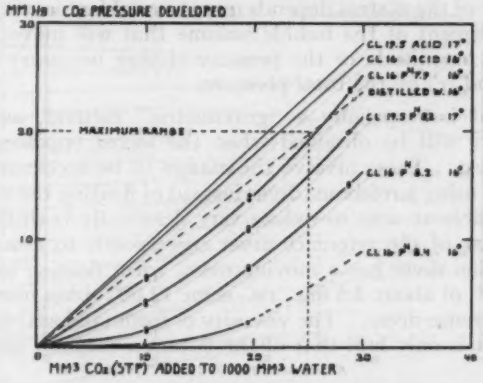


FIGURE 6. Pressure developed by adding known amounts of CO_2 to fresh water and sea water of varying pH and chlorinity.

down to a negligible amount. With less favorable gas-to-liquid ratio, and for special purposes, it may, however, be desirable to add a buffer to the sea water (Zeuthen, 1950).

The maximum amount of oxygen that can be used up in a chamber containing oxygen equilibrated water corresponds to some 2–3% of the water volume. The maximum CO_2 that can be metabolically produced at *RQ* 1 is then the same, namely around 20–30 volumes per 1000 water. The pressure effect of this can be read on Figure 6, and corresponds to 20 mm. Hg. It will usually be less than 10 mm. or corresponding to about 1% CO_2 at atmospheric pressure.

C. Diffusion characteristics of the system

This system, like all others, depends upon diffusion equilibrium between the gas and the water phase. The faster equilibrium is regained after a disturbance, the faster the system will be able to follow quick changes in respiration. Since the diffusion distances are very short in all directions (maximum 0.3 mm.) and

the volumes involved are very small, there is an exceptionally rapid tension equilibrium within the system.

This can easily be determined empirically. A blank is set up without respiring material and a 10–15 minute run is made in which the flotation pressure is accurately maintained and plotted every minute. A major disturbance is now introduced by putting 10 centimeters Hg pressure on the diver until within a minute the bubble has almost disappeared by dissolving into the water. The diver is again brought to flotation, and faster than it can be plotted, it regains its former flotation pressure within a half millimeter Hg. In other words, even major disturbances, involving the new generation of the gas bubble from the dissolved gas, are repaired within 30 seconds. Clearly, therefore, we can accurately record even very rapid respiratory changes with very little damping effect (Linderstrøm-Lang, 1946).

D. Sensitivity

The sensitivity of the system depends upon several factors: (a) on the smallest increment or decrement of the bubble volume that will move the diver up or down, (b) on the magnitude of the pressure change necessary to produce this volume change, and (c) on the total pressure.

a. The method is essentially a "gravimetric" method, and therefore the greatest sensitivity will be obtained when the forces opposing the buoyancy changes are smallest. These involve the masses to be accelerated and the frictional forces. By using a reference diver instead of floating the whole respiratory chamber it is possible at once to reduce very drastically both the mass and the friction. The mass of the reference diver corresponds to around 150–200 $\mu\mu$l. The micro Cartesian diver has a moving mass, when floating in the medium of specific gravity 1.3, of about 2.5 mg., *i.e.*, some 12,000 times more mass to accelerate than the reference diver. The viscosity of fresh (and sea) water for floating the reference diver is only half that of the flotation medium used for Cartesian divers.

b. The pressure necessary to convey a volume change to the diver gas depends upon the number of menisci to be moved or deformed. The reference diver has one meniscus, whereas the micro diver involves four. The present size reference diver can be held within about 0.2 millimeter Hg, and we have not so far tried a water manometer. During oxygen consumption, when the flotation pressure is steadily decreasing, the accuracy of defining the flotation pressure undoubtedly goes down, somewhat in proportion to the rate of pressure change.

c. The sensitivity bears a direct relation to the total pressure at which the diver is operated. This is apparent if we consider the least molar amount of gas added or withdrawn that we can measure. The resulting volume change will depend upon the ratio of the amount withdrawn to the total amount present in the system. The sensitivity related to molar amounts of gas, therefore, increases in an inverse proportion to the pressure. The higher sensitivity at lower pressures is clearly felt in the manipulation of the syringe.

E. Stability in time

The stability of the flotation pressure is measured by blank runs. It depends on several factors, the most important ones being: (1) constancy of the tem-

perature of the water bath and of the manometer, and (2) diffusion of gas into the chamber from the water trapped in the capillary space between the rod and the chamber. Other sources which, especially during respiratory runs, could cause a shift of the base line would be (3) changes in surface tension, (4) changes in water vapor tension and density of the medium, (5) changes in area of bubble attachment to the diver, with consequent change in diameter and pressure.

On Figure 7 are given curves of the stability of the flotation pressure as found in several series of blank runs in fresh water, sea water and some media. The stability of the flotation pressure seems largely independent of the chamber size.

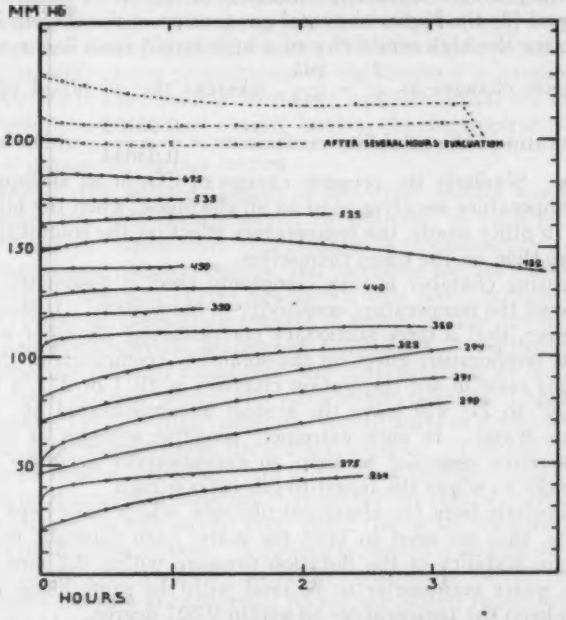


FIGURE 7. Stability of flotation pressure in blank runs. The number at the end of each curve plus 70 gives the flotation pressure in mm. Hg at the beginning of the blank run.

The stability in absolute volume units can therefore be taken as nearly proportional to the chamber size, *i.e.*, the factor F (p. 164). On the chart the factors actually went from 0.7 to 10, *i.e.*, one mm. Hg corresponded to from 0.7 to 10 $\mu\mu$l.

1. Effect of temperature changes

From equation 1 we may calculate the temperature sensitivity of the system by knowing the amount by which the gas bubble, the solubility coefficient, the water vapor tension and the surface tension change with temperature. If we move from 20° to 21° C., α_{O_2} changes from 0.03102 to 0.03044, α_{CO_2} from 0.878 to 0.854, water vapor tension from 17.54 to 18.65 mm. Hg, and bubble pressure due to surface tension from 31.18 to 31.11 mm. Hg. If the bubble volume is

180 $\mu\mu$l and the liquid volume is 20000 $\mu\mu$l and the gas (O_2) is at 760 mm., this rise in temperature will change the pressure by 13.3 mm. Hg. A hundredth of a degree will change the pressure near to 0.13 mm. Hg.

It may similarly be calculated that when all the O_2 of the system (802 $\mu\mu$l) has been used up and 802 $\mu\mu$l CO_2 have been produced, the resultant total pressure will be 20.8 mm. Hg at 20°. A one degree rise will then produce a 2.1 mm. increase in pressure, and 0.01° therefore 0.02 mm. increase.

If the system were all a moist gas phase it can similarly be shown that the same rise in temperature would raise the flotation pressure only 4 mm. We see thus that the temperature sensitivity increases: (1) the more liquid there is compared to gas, and (2) the higher the total gas tension of the system is.

The reason for the high sensitivity of a high liquid ratio lies in the fact that the all gas phase changes as $\frac{T}{T_1} = \frac{294}{293}$, whereas the all liquid phase tension changes as the ratio of the solubility coefficients $= \frac{0.03102}{0.03044}$, which is a 5.6 times greater change. Similarly the pressure change of CO_2 in an all liquid system is 8.2 times as temperature sensitive as in an all gas phase, when the initial pressure is the same. In other words, the temperature effect on the solubility coefficients is much greater than on the gases themselves.

A compensating chamber is very commonly used in gasometric methods in order to cut down the temperature sensitivity of the system. It should be borne in mind, however, that a large stationary compensating chamber will not compensate for the temperature effect on the solubility coefficients of the gases. If the liquid-to-gas ratio in the respiration chamber is 10/1 or 2/1, a temperature change from 20° to 21° will leave the system uncompensated by, respectively, 40 and 10 mm. water. In such extremely sensitive systems as the Cartesian diver, it is therefore essential to keep an exceptionally accurate temperature control, especially so where the liquid-to-gas ratio is high.

We may conclude from the above calculations, which have been verified also by experiments, that we need to keep the water bath constant to within 0.01 degree to obtain stability of the flotation pressure within 0.2 mm. Hg. If we were to use a water manometer to be read with the same linear accuracy we would have to keep the temperature to within 0.001 degree.

It can easily be calculated that the mercury barometer must be kept within 2° C. in order to match the temperature sensitivity of the water bath, *i.e.*, $\pm 0.01°$.

2. Diffusion of gases from the sleeve

The blank curves (Fig. 7) are arranged in a sequence, downwards according to the initial pressure reading, which is given in mm. Hg at the end of each curve. It will be seen that the lower curves, with a low flotation pressure, all show an initial rise, which in the first hour may amount to as much as 10 mm. Hg. This increase in flotation pressure diminishes, or disappears, as the flotation pressure approaches atmospheric pressure. The rise is due to gas diffusing in from the water trapped at one atmosphere's pressure in the capillary sleeve between the rod and the chamber wall. This can be proved by evacuating the loaded chamber for several hours, so that finally the sleeve water gets a low gas tension. When,

now, the flotation pressure is established, gas will diffuse back into the sleeve water from the chamber, producing a slight decrease in the flotation pressure (see the three upper curves).

We have not been able to demonstrate any measurable diffusion of gas *through* the sleeve. Even such drastic measures as changing the outside water from vacuum extracted to fully saturated water leave the flotation pressure undisturbed. Evidently diffusion through the capillary sleeve is exceedingly slow.

The pumping action produced by the slight volume changes in the bubble while adjusting the flotation pressure is much too small to effect any water renewal in the sleeve. It has so far been impossible even with gross changes in the bubble volume by vacuum to pump any dyed outside liquid into the chamber.

As the pressure during a run decreases the sleeve presumably continues to yield a small amount of gas proportional to the decrease in pressure. This will make a slight error in the chamber factor. Considering that the chamber factor and the cell volume are not very exactly known, the sleeve error is unimportant.

3. Effect of changes in surface tension

Any change of the surface tension of the diver bubble would change the bubble pressure, and this change would be reflected as such on the flotation pressure. During an experiment the medium is continuously changing with respect to gas tensions, pH, and organic and inorganic solutes due to the presence of the organism. Ferguson (1914) has shown that one atmosphere of CO_2 gas has very little effect on the surface tension of water, so that the maximum effect from CO_2 in our system would be entirely negligible. A negligible effect would also be produced by relevant changes in the concentrations of NaCl, KCl, urea, glycerine, sucrose, sodium phosphate, ammonium chloride (Landolt-Börnstein, 1923; McClendon, 1917). High molecular constituents of urine, bile and plasma may, however, significantly lower the surface tension. It would be of no significance for the method if a medium had a different surface tension from that of water, as long as it remained constant. It would not matter, either, if it showed a slow linear change, as this would only affect the factor converting pressure to absolute units of O_2 consumption. A sudden release of a surface acting substance (*e.g.*, protein) could give a sudden parallel displacement of the rate curve, but would then leave the rate unchanged. As surface acting substances are concentrated in the surface, they tend to exercise near their full action already at minute concentrations. If such substances were released periodically, it seems therefore likely that they would lead to only small periodic changes in the surface tension.

4. Changes in water vapor tension and density of medium

Only a change in the water vapor tension during the experiment will affect the results. A gradual change would be of no consequence, as both the absolute rate and the amount of respiring material would be less well known. Measurable sudden changes in water tension are even more unlikely than sudden changes in surface tension, and would tend to be counteracted by the density change.

5. Changes in bubble diameter

There is a possibility of a pressure change in the bubble if it changes its adhesion surface to the diver. This could happen if it does not fully occupy the whole cut surface.

The salt water diver has an oblique upper surface with no boundaries set for the bubble, which therefore could be assumed to vary in its attachment surface, and hence radius and pressure. In the experiments referred to on page 168, where the diver was subjected to gross transitory pressure changes, no changes in the final flotation pressures were detected, and hence there were no measurable changes in bubble radius. For tension determinations where it is necessary to generate the bubble twice, the diver is so long that the bubble will always completely occupy the cut surface.

III. Procedure

1. Preparation of sterile material and chamber

It seems that bacterial contamination is a more conspicuous menace in this method than in macro methods. It is essential always to keep this in mind and try to avoid insidious errors from this source. Nutritive media especially, such as, for instance, hay tea or even sea water, must be very carefully sterilized and handled. Sea water was filtered through a porcelain candle (Selas, porosity 015) and then heated overnight in a thermostat of 70° C., allowed to stand at room temperature the next day, and then heated again to 70° C. The cool water was shaken with air and poured into two or three covered sterile Syracuse dishes.

All instruments and glass tubes, tips, vials, dishes and respiration chambers with rods must be boiled or autoclaved immediately before use.

The cell is transferred by means of a braking pipette into the water or medium of one dish, where it is allowed to sink or swim about. The pipette is rinsed several times in sterile water and the cell similarly transferred to two more dishes.

2. Loading the chamber (Fig. 8)

The medium is poured into a tube which is placed in a beaker of ice water and bubbled with moistened oxygen. The tube is kept loosely covered. After half an hour there will be enough oxygen in the water. The respiration chamber is "threaded" up on the suction tip, where it is held by the suction. The tube with bubbler is lifted up submerging the respiratory chamber, which thereby is filled and rinsed. The chamber is gripped by forceps while completely submerged, and the whole assembly is lowered as one piece down from the suction tip, leaving the respirometer chamber filled. The chamber is removed from the liquid and rolled up under a hook on the rubber stopper that serves as a chamber holder. It is imperative that there be no air bubbles sticking to the walls of the chamber. This is ascertained under the dissecting microscope. The cell is now transferred to a dish where it is allowed to sink through a centimeter or so of sterile medium and is then transferred into the opening of the chamber with a fine braking pipette. If the cell readily sinks, the chamber holder is put on its flat end. If the cell swims or does not freely sink to the closed end of the chamber, the chamber is placed in a simple cork centrifuge which is run very gently from a Variac. The chamber is then transferred back to the holder under the binocular.

At this point the diver is cut as described (p. 161), or picked up with a braking pipette from its storage drop.[9] While still on the knife tip it is transferred to the

[9] The diver filament will not stand heat sterilization. It may be wiped off with alcohol and water, or only water.

meniscus of the chamber where it will adhere to the surface. Using a fine needle, it is pushed into the liquid, unless deposited by a pipette. The stopper is held with chamber opening up and a slight tapping is usually enough to bring the diver down to the bottom next to the cell.

The rod is now rinsed off in the same medium from which the cell was taken. One of the drops adhering to it is touched to the chamber opening so the meniscus is convex and the solid rod is now inserted. In case the cell is a swimming organism like Paramecium, it may be quite a trick to get the rod in, using a combination of centrifugation and slight advances every time the animal moves in until the rod is finally seated. It is possible to some extent to feel how the rod should be rotated to seat deepest. Any quick pull back must be avoided,

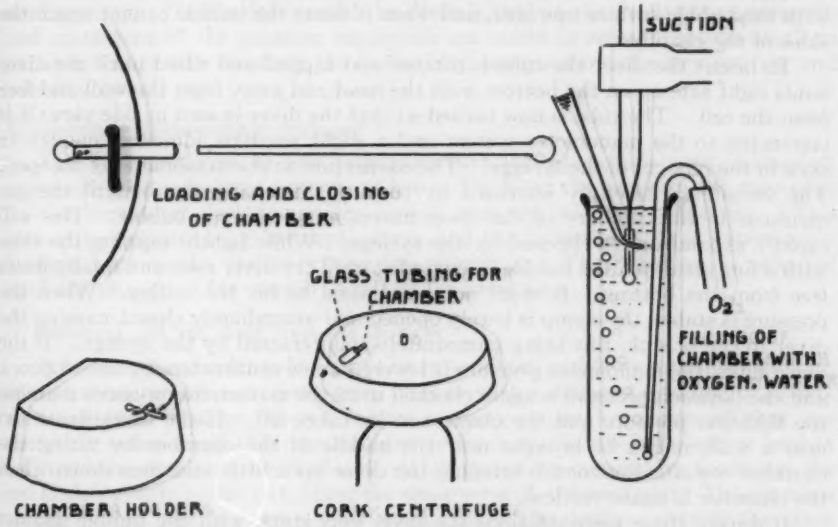


FIGURE 8. Operations involved in loading the chamber with oxygenated medium, cell and diver.

as this cavitates a bubble onto the diver and a positive flotation pressure may result.

The chamber, rod down, is now put into the chamber container, which is filled two thirds with the medium. The bubble fiber (p. 160) is put in and the container clamped in place on the water bath.

3. Measurement of chamber

At this time it is convenient to measure the chamber height and diameter by the ocular micrometer, and also the height of the diver from the base to the opening of the capillary hole. If a measurable cell is being used, like an egg cell, its diameter is also measured. It is often possible at once to calculate the chamber volume from the dimensions by using the chart mentioned on page 164, and from another chart (p. 175) read off the factor which translates mm. Hg to $\mu\mu l$ O_2.

Especially if working with an assistant, one can then usually have the readings calculated and plotted during the run.

4. Floating the reference diver (Fig. 3)

For this step to succeed, it is necessary that the chamber and the rod be absolutely clean so the diver will not stick. With fresh water the walls are usually hydrophilic, a straight cut diver can be used and the flotation is easily accomplished. In sea water the walls become hydrophobic and flotation cannot be accomplished unless the bubble is kept from touching the glass walls. There are two precautions taken to make flotation possible: (1) shape of the diver; (2) orientation of the diver before generating the bubble.

The diver is shaped so that when it sinks and hits the bottom it tends to fall with the bubble surface upwards, and when it floats the bubble cannot touch the sides of the chamber.

To orient the diver the tube is rotated and tapped and tilted until the diver lands right side up on the bottom with the head end away from the wall and free from the cell. The tube is now turned so that the diver is seen in side view; it is connected to the manometer system and a slight vacuum admitted, enough to suck in the plunger of the syringe. The connection to the manometer is clamped. The vacuum is carefully increased by turning the syringe knob until the gas meniscus in the capillary of the diver moves out making a bubble. This will rapidly expand unless checked by the syringe. While lightly tapping the tube with a fine glass rod, the bubble is expanded until the diver rises and finally floats free from the bottom. It must not be allowed to hit the ceiling. When the pressure is stable, the clamp is barely opened and immediately closed, causing the diver to rise or sink, this being immediately counteracted by the syringe. If the diver sinks, the manometer pressure is lowered a few centimeters by the stopcock and the flotation pressure is again checked until the manometer pressure matches the flotation pressure and the clamp can be taken off. If the diver floats too near a wall, it can be brought near the middle of the chamber by tilting the chamber towards horizontal, bringing the diver up a little and then down when the chamber is again vertical.

If during these manipulations the diver gets stuck with the bubble against the bottom, top or side walls, pressure is put on to near atmospheric. The syringe plunger is pulled half way out and the clamp tightened. By applying overpressure with the syringe the bubble is made to disappear into the diver capillary and the diver is again free and can be re-oriented before a new flotation is attempted. If the diver itself sticks, it can usually be shaken loose if the chamber tube is removed from the water bath and given a violent shake down.

If the bubble inadvertently should dissolve completely, a new one usually cannot be formed by simple evacuation. There is still one chance left to save the run, namely, by cavitating a bubble onto the diver. To do this the chamber is removed from the tube and is held submerged in a dish of the medium. The rod is barely loosened very carefully and immediately pushed back in. This cavitates a bubble onto the diver which then often can be made to float. It is immaterial where the bubble forms on the diver. The flotation pressure is often high as a result of this procedure, sometimes even higher than atmospheric pressure. In such case air must be blown into the barometer flask and the

syringe must be manipulated with two hands, one continually pressing the barrel in, while the other regulates the motion.

5. Recording of the flotation pressure

With the reference diver afloat, the measurements can start, and readings can be taken at any suitable interval. The ocular scale is oriented in a vertical position, and the middle line of it is placed a little above the middle of the chamber. The distance between this line and the bottom of the chamber is maintained throughout the run, and the bubble is held so that the top of it touches the ocular line.

For most accurate readings, especially if close time intervals are desired, it is best to have an assistant start calling off every five seconds a half minute before reading time. During this period the diver is held at the mark very accurately and excursions of the pressure regulation are made as smooth and as small as possible. It is wise to check the water bath temperature at every reading and note it down.

6. Re-setting of manometer pressure

The syringe serves as fine regulation and its capacity is soon exhausted, especially when the total pressure is low. To re-set the pressure the stop-cock on the barometer flask is rapidly turned past the vacuum connection. This lowers the pressure by a few centimeters, and the diver rises. It is immediately brought back to the line by letting the plunger in. The procedure is repeated until the plunger is far enough in to last for some time.

7. Removing cell and diver from the chamber

It is sometimes useful to be able to remove the cell and diver from the chamber, so that the same run can be repeated later or with a different medium, or it may be desirable to measure the cell volume or other properties of the cell that was used. In order to do this, the chamber is taken out of the tube and is held in a Petri dish containing the medium. With the sleeve covered by the liquid the rod is very gently pulled out, filling the chamber with the medium. The chamber is then placed, opening down, in a small dish of the medium, letting the diver and the cell fall out.

8. Calculation of results

It is customary in micro respirometry to recalculate the readings as increasing from the first one, which is called zero, by subtracting all the subsequent readings from the first. In a detailed study all these figures, showing the change in pressure as a function of time, must be recalculated to give $\mu\mu l$ O_2 consumed. This is done by first calculating the liquid volume as described on page 164 and then using the formula (8). If the diameter of the diver filament is kept constant and the diver length reasonably constant, a great deal of calculation can be saved by computing the factor for four situations, using two different chamber volumes, of, say, 20,000 $\mu\mu l$ and 100,000 $\mu\mu l$, and two different diver lengths, one with height of the diver equal to the diameter and the other equal to one and a half times the diameter. A graph is prepared with the factors on the ordinate and V_L on the abscissa. The four points are plotted and the pairs connected with

parallel lines. In the relevant region the function (equation 8) is very nearly a straight line, and the factor is found by interpolation.

This chart can of course only be used in series where the temperature, salinity and diver diameter are kept the same.

We are greatly indebted to the officers of the William F. Milton Fund for making this development possible.

We wish to express our appreciation to Dr. A. Baird Hastings for constant encouragement and advice. He has forcefully backed the project from its first vague beginning. Miss Barbara Orski has contributed several blank runs, and Mrs. Susan I. Scholander has given much technical and secretarial help during the later stages of the development. We wish to convey to these ladies our affectionate thanks.

Summary

1. A method for micro respiratory studies is described with a sensitivity down to 0.2 millionth of a cubic millimeter and a stability of 10–0 millionths of a cubic millimeter an hour. It is basically a constant volume method, where a minute reference diver is introduced into the chamber together with the cell. A bubble is generated on the diver until it just floats. The change in flotation pressure multiplied by a factor of known constants gives the oxygen consumption. The CO_2 is effectively held in solution by the high liquid-to-gas ratio. The system has been used to study the respiration of single protozoans and to follow in detail the oxygen consumption during cleavage of single eggs of several species, which will be reported elsewhere.

2. A new micro method is described for direct determinations of gas tensions in liquids using the reference diver.

LITERATURE CITED

Andresen, N., H. Holter and E. Zeuthen, 1944. The respiration of syncytia, formed by abnormal development of Ciona eggs. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **25**: 67–85.

Boell, E. J., J. Needham and V. Rogers, 1939. Morphogenesis and metabolism: Studies with the Cartesian diver ultramicromanometer. I. *Proc. Roy. Soc. London, ser. B.*, **127**: 322–356.

Claff, C. L., 1947. "Braking" pipettes. *Science*, **105**: 103–104.

Dixon, M., 1943. Manometric methods. Sec. ed. Macmillan Co., New York.

Ferguson, A., 1914. On the surface-tensions of liquids in contact with different gases. *Phil. Mag., ser. 6*, **28**: 403–412.

Holter, H., 1943. Technique of the Cartesian diver. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **24**: 399–478.

Holter, H., and E. Zeuthen, 1944. The respiration of the egg and embryo of the ascidian, *Ciona intestinalis* L. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **25**: 33–65.

Krogh, A., 1911. On the hydrostatic mechanism of the Corethra larva with an account of methods of microscopical gas analysis. *Skandinavisches Arch. Physiol.*, **25**: 183–203.

Landolt-Börnstein physikalisch-chemische Tabellen, 1923. Fünfte umgearbeitete und vermehrte Auflage. Herausgegeben von Dr. Walther A. Roth und Dr. Karl Scheel. Berlin, vol. 1.

Linderstrøm-Lang, K., 1937. Principle of the Cartesian diver applied to gasometric technique. *Nature*, **140**: 108.

Linderstrøm-Lang, K., 1942. Approximate solution of certain diffusion problems in liquid systems. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **24**: 249–280.

LINDERSTRØM-LANG, K., 1943. On the theory of the Cartesian diver micro respirometer. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **24**: 333–398.

LINDERSTRØM-LANG, K., 1946. Periodic metabolism and diffusion. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **25**: 229–272.

LINDERSTRØM-LANG, K., AND D. GLICK, 1938. Micromethod for determination of choline esterase activity. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **22**: 300–306.

LINDERSTRØM-LANG, K., AND H. LANZ, JR., 1938. Studies on enzymatic histochemistry. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **21**: 315–338.

MCCLENDON, J. F., 1917. Physical chemistry of vital phenomena. Princeton Univ. Press, Princeton, N. J.

PETERS, J. P., AND D. D. VAN SLYKE, 1932. Quantitative clinical chemistry. Volume II. Methods. Williams and Wilkins Co., Baltimore.

SVERDRUP, H. U., M. W. JOHNSON AND R. H. FLEMING, 1942. The oceans. Prentice-Hall, Inc., New York.

ZEUTHEN, E., 1943. A Cartesian diver micro respirometer with a gas volume of 0.1 µl. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **24**: 479–518.

ZEUTHEN, E., 1946. Oxygen uptake during mitosis: Experiments on the eggs of the frog (*Rana platyrrhina*). *C. R. Travaux Lab. Carlsberg, sér. chim.*, **25**: 191–228.

ZEUTHEN, E., 1950. Respiration during cell division in the egg of the sea urchin *Psammechinus miliaris*. *Biol. Bull.*, **98**: 144–151.

RESPIRATORY STUDIES OF SINGLE CELLS. II. OBSERVATIONS ON THE OXYGEN CONSUMPTION IN SINGLE PROTOZOANS [1]

P. F. SCHOLANDER, C. L. CLAFF AND S. L. SVEINSSON

Department of Biological Chemistry and Laboratory for Surgical Research, Harvard University Medical School, Boston, Mass.

During the development of the reference diver technique (Scholander, Claff and Sveinsson, 1952) for studying single cell respiration, many measurements were made on single protozoans. We are here presenting observations on (1) the relation between cell volume and respiratory rate as determined over a thousandfold range in size, using individual cells from three species of protozoans, and (2) the metabolic change occurring at excystment and encystment of *Bresslaua*.

Material and Methods

Respiratory measurements

A single animal was pipetted from the stock culture, usually hay tea with a few wheat grains, and transferred with a minimum amount of water into a dish containing sterile Ringer solution diluted 50 times. Two to three additional transfers were made, where the cell was allowed to swim through, or was moved through, relatively enormous amounts of sterile solution. If any bacteria were left clinging to the cell the inorganic medium would hardly support any appreciable growth during the time of the experiment. All measurements were made at 25° C. and each run lasted 60–70 minutes. Our runs confirm the findings by Amberson (1928) that the oxygen rate stays constant in spite of large changes in the oxygen tension. After the run the cell was transferred to a dish and the cell volume could subsequently be measured.

Measurement of cell volume (Fig. 1, A and B)

The cell volumes were measured by squeezing the cells between two glass surfaces spaced at a known distance, and tracing the surface contour. Mast and Fowler (1935) and Pèterfi and Maleci (1938) used a similar method of deforming the cells, by sucking them into a capillary tubing of known diameter and measuring the length.

For the measurements two brass wire frames 1 × 2 cm. are prepared. Stretched across the frame on the under side are two platinum filaments [2] of accurately known diameter. One frame had filaments of 19 μ, the other of 38 μ. A 10 × 5 mm. piece of a microscope slide serves as a thick and heavy coverslip. The frame is

[1] This work was supported by a grant from the William F. Milton Fund.

[2] Obtained from Baker and Co., Newark, N. J.

placed on a microscope slide, and the cell in a small drop of water is placed between the filaments. The cover glass is carefully put on. Dust must be carefully avoided on the spacer filaments. A paramecium can move about slowly even when squeezed flat to about one fifth or one sixth of its normal diameter. When at rest its circumference is rapidly traced through an Abbé prism onto a piece of paper which has a known weight per surface area. The cell tracing is cut out and weighed and the cell volume is calculated from the surface area and the thickness. In one

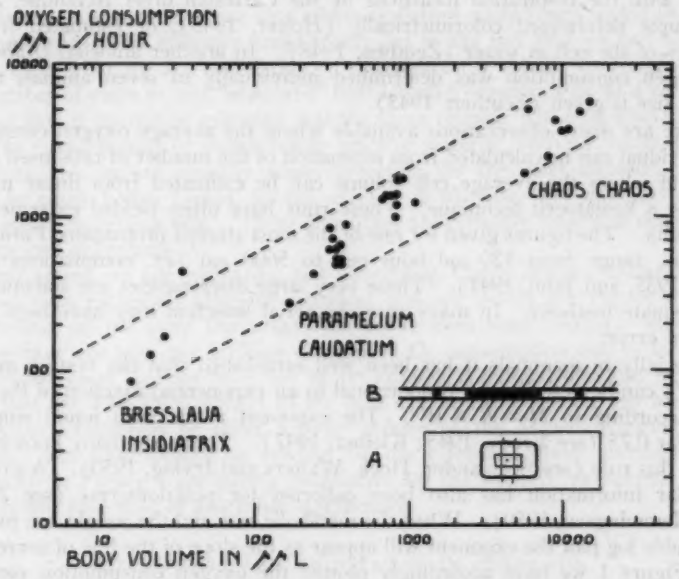


FIGURE 1. Ratio of oxygen consumption to cell volume, both determined in the same individual cell. The parallel lines indicate the general trend of the data. The slope of the lines is the exponent n of the equation $M = K \times W^n$, where M is the oxygen consumption, K is a proportionality constant and W is the weight. We attach no significance to the empirical value of n fitting these data. Insert A. Method for measuring cell volume by squeezing cell to a known thickness under a cover glass resting on two spacing filaments. Insert B. Cross section showing spacing filaments and flattened cell between the glass surfaces. One $\mu\mu l$ = one millionth of one mm^3.

species of *Paramecium* several measurements on the same individuals gave an agreement within ± 6%.

The amoeba *Chaos chaos*, which cannot be flattened nearly as much as a paramecium without rupturing, was squeezed between thicker filaments or even under the coverslip of a blood cell counting chamber. In order to succeed in the measurement it is necessary to tease the amoeba to contract by tapping the glass and trace it quickly before the protoplasm begins to flow out at the edges. In the amoeba the volume measurements are probably not better than ± 10%.

Relation of Size to Oxygen Consumption in Some Protozoans

Using the reference diver technique we have determined the oxygen consumption in a series of cells of different species and size. The absolute accuracies obtained are estimated to be within about ± 10% for the smaller species and ± 5% for the larger species.

There seem to be very few cases where metabolic rate and cell volume are given for the same cell. Holter and Zeuthen (1948) give such data for amoebae (*Chaos chaos*), with the respiration measured by the Cartesian diver technique, and the cell volume determined colorimetrically (Holter, 1945), or estimated from the buoyancy of the cell in water (Zeuthen, 1948). In another amoeba, *Difflugia* sp., the oxygen consumption was determined individually in seven animals and the average size is given (Zeuthen, 1943).

There are many observations available where the average oxygen consumption per individual can be calculated from estimation of the number of cells used in mass runs, and where the average cell volume can be estimated from linear measurements or a hematocrit technique. These runs have often yielded extremely variable results. The figures given for one of the most studied protozoans, *Paramecium caudatum*, range from 120 $\mu\mu$l/hour/cell to 5600 $\mu\mu$l (*cp.* compilations by von Brand, 1935, and Jahn, 1941). These very large discrepancies are sometimes due to inadequate methods. In many cases bacterial infection may have been a large source of error.

Especially in mammals it has been well established that the resting metabolic rate (M) can be described as proportional to an exponential function of the weight (W), according to $M = K \times W^n$. The exponent n has been found empirically to be near 0.75 (see Brody, 1945; Kleiber, 1947). There are many known exceptions to this rule (see Scholander, Hock, Walters and Irving, 1950). A great deal of similar information has also been collected for poikilotherms (see Zeuthen, 1947; Hemmingsen, 1950). When the metabolic rate and the weight are presented on a double log plot the exponent will appear as the slope of the line of correlation.

In Figure 1 we have accordingly plotted the oxygen consumption versus the volume of the cell, and in this material of a thousandfold range in size, the exponent (slope) is low, about 0.55. If the dotted parallel lines are extended down to the size of bacteria, we will hit *Bacterium coli* at a cell volume of 0.5–1 μ^3 and an O_2 consumption of 0.1–1 $\mu\mu$l/cell/hour (Martin, 1932; Huntington and Winslow, 1937; Hershey and Bronfenbrenner, 1938), but we will be about ten times too high for *Photobacterium phosphorescens*, which, with a volume of 1.7 μ^3, has only 0.06 $\mu\mu$l O_2 consumption (Harvey, 1928). If we start at the upper end, the data on *Chaos chaos* by Holter and Zeuthen (1948) fit well between the lines and so do the data on *Difflugia* when the shell is disregarded (Zeuthen, 1943). The oxygen consumption of *Tetrahymena geleii*, with 15–24 $\mu\mu$l cell volume, has been found by several investigators to lie between the lines (see Ormsbee, 1942; Pace and Lyman, 1947), but it may rise up to three to four times higher than the upper line when given suitable substrate. *Astasia klebsii*, on the other hand (Pringsheim, 1936; von Dach, 1940, 1942), was found about twenty times lower than the *"coli-chaos"* line when starved, but on the line when fed on acetate (cell volume about 5 $\mu\mu$l, O_2 consumption 2–4 $\mu\mu$l/hour). *Sarcina*, with a cell volume of near 2 μ^3, was found by

Gerard and Falk (1931) to use about 0.007 $\mu\mu$l/cell/hour, which places it about 100 times below the lines.

It seems that these samples of data from careful investigations point towards a very considerable variation in the relation between size and metabolic rate in the range of single cell organisms. It would appear impossible to fit the data into any simple rule, such as the "mouse to elephant" curve (Benedict, 1938), or even the "beech tree to egg of silkworm" curve discussed by Hemmingsen (1950). When interpreting the available data on single cells this author arrives at an exponent near 1, whereas according to our data and compilation many forms would fit a much lower exponent. The difficulty undoubtedly lies in the fact that we can only very poorly define what we are correlating. Although on a volume or weight basis a large number of more or less "standard" cold-blooded organisms do fit a certain exponential line, it cannot be expected that the same relation will hold also for forms that carry vast amounts of metabolically inactive material, such as corals, jellyfish, eggs, trees, etc., unless possibly some correction for the inert material could be made. In many forms (seeds, spores, etc.) it is likewise very difficult to define what is meant by resting rate. These difficulties are certainly no less in dealing with the single cell organisms.

In our series, we are comparing fast swimming, highly active *Paramecium* and *Bresslaua* with extremely sluggish amoebae, and we would not expect that both would belong on the same line unless the active ones were resting. In our material we have not seen any striking changes in O_2 consumption due to locomotion in the *Paramecium*, but we have not as yet studied it extensively, and we do not believe that the 200–300% spread in the data can be explained on this basis. To what extent locomotion or other activity influences the oxygen consumption in protozoans can undoubtedly be studied by the reference diver technique. The lines drawn in Figure 1 are purely descriptive for the material presented, and are at the present time void of any other significance.

Excystment and Encystment in Bresslaua

Bresslaua insidiatrix (Claff, Dewey and Kidder, 1941) will form a protective resting cyst if left long enough in pure water. It will start to circle and to eject a protective cyst shell in which it keeps turning for several hours until it finally stops. It can stay dried up as an undifferentiated, spherical, resting cyst for years. If this cyst is placed in hay tea it will start to differentiate and will excyst within an hour or so. A pulsating vacuole is formed, the animal starts circling inside the cyst shell and finally breaks through.

Gregg (1947) measured the effect of encystment on this organism, using three individuals simultaneously in a micro modification of the Warburg respirometer. He observed a 50% decrease in the oxygen consumption when the cells encysted. The rate of oxygen consumption of the free swimming cell was found to be about 10,000 $\mu\mu$l/cell/hour at a cell volume which, estimated from the linear dimensions given, cannot have been more than 500 $\mu\mu$l. This gives about ten times higher rate than indicated by the present method.

The respiratory events at excystment and encystment were followed in the respirometer, using single cysts or cells (Fig. 2). The O_2 consumption in resting

cysts was immeasurable by this method. In order to effect excystment the cyst was placed for five minutes in sterile hay tea and then transferred several times in sterile 1/50 dilute Ringer solution in which it was run. Hay tea is a better medium for excystment but was found too difficult to maintain sterile.

After the activation by hay tea there is a steady increase in oxygen consumption, and at the moment the organism breaks through the cyst there is a definite but often quite small rise. It is conceivable that this rise is merely due to the elimina-

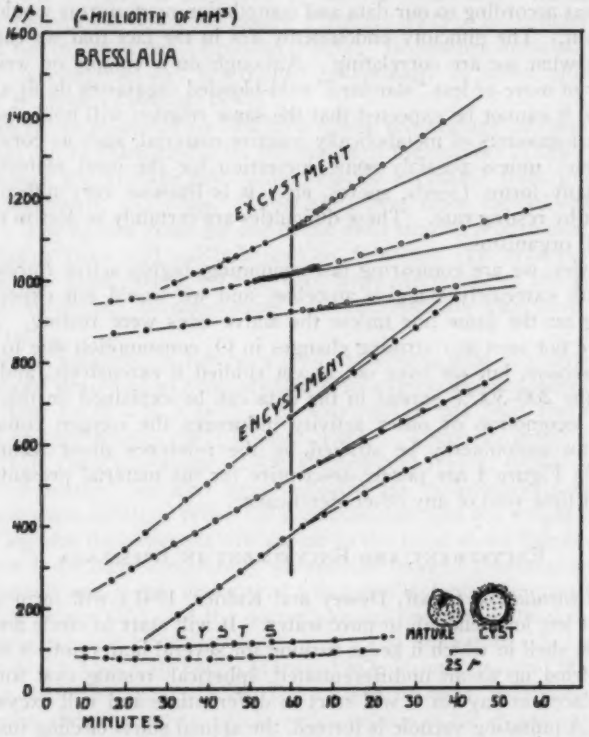


FIGURE 2. Oxygen consumption at excystment and encystment in single individuals of *Bresslaua insidiatrix*.

tion of the shell, which would act as a limiting factor for the oxygen diffusion at the high metabolic rate of the fully developed cell.

In Figure 2 three runs are given showing the reverse effect on the oxygen consumption when the cells are encysting.

We wish to express our gratitude to Dr. A. Baird Hastings for much helpful advice during the work and to the officers of the William F. Milton Fund for supporting this investigation.

SUMMARY

1. Observations are reported on the oxygen consumption of single individuals of four species of protozoan using the reference diver technique.

2. The relation of oxygen consumption to cell volume is given for a series of individual protozoans covering a thousandfold range in size, and the change in oxygen consumption following excystment and encystment in *Bresslaua* has been recorded.

LITERATURE CITED

AMBERSON, W. R., 1928. The influence of oxygen tension upon the respiration of unicellular organisms. *Biol. Bull.*, **55**: 79–91.

BENEDICT, F. G., 1938. Vital energetics. Carnegie Inst. Washington Publ. no. 503.

BRAND, T. VON, 1935. Der Stoffwechsel der Protozoen. *Ergeb. Biol.*, **12**: 161–220.

BRODY, S., 1945. Bioenergetics and growth. Reinhold. New York.

CLAFF, C. L., V. C. DEWEY AND G. W. KIDDER, 1941. Feeding mechanisms and nutrition in three species of *Bresslaua*. *Biol. Bull.*, **81**: 221–234.

DACH, H. VON, 1940. Factors which affect the growth of a colorless flagellate, *Astasia klebsii*, in pure cultures. *Ohio J. Sci.*, **40**: 37–48.

DACH, H. VON, 1942. Respiration of a colorless flagellate, *Astasia klebsii*. *Biol. Bull.*, **82**: 356–371.

GERARD, R. W., AND I. S. FALK, 1931. Observations on the metabolism of *Sarcina lutea*. I. *Biol. Bull.*, **60**: 213–226.

GREGG, J. R., 1947. A microrespirometer. *Rev. Sci. Inst.*, **18**: 514–516.

HARVEY, E. N., 1928. The oxygen consumption of luminous bacteria. *J. Gen. Physiol.*, **11**: 469–475.

HEMMINGSEN, A. M., 1950. The relation of standard (basal) energy metabolism to total fresh weight of living organisms. *Rep. Steno Memorial Hosp. Nordisk Insulinlab.*, **4**: 7–58.

HERSHEY, A. D., AND J. BRONFENBRENNER, 1938. Factors limiting bacterial growth. III. Cell size and "physiological youth" in *Bacterium coli* cultures. *J. Gen. Physiol.*, **21**: 721–728.

HOLTER, H., 1945. A colorimetric method for measuring the volume of large amoebae. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **25**: 156–167.

HOLTER, H., AND E. ZEUTHEN, 1948. Metabolism and reduced weight in starving *Chaos chaos*. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **26**: 277–296.

HUNTINGTON, E., AND C-E. A. WINSLOW, 1937. Cell size and metabolic activity at various phases of the bacterial culture cycle. *J. Bact.*, **33**: 123–144.

JAHN, T. L., 1941. Respiratory metabolism. *In:* Calkins, G. N., and F. M. Summers, Protozoa in biological research. Columbia Univ. Press, New York, chapt. 6, pp. 352–403.

KLEIBER, M., 1947. Body size and metabolic rate. *Physiol. Rev.*, **27**: 511–541.

MARTIN, D. S., 1932. The oxygen consumption of *Escherichia coli* during the lag and logarithmic phases of growth. *J. Gen. Physiol.*, **15**: 691–708.

MAST, S. O., AND C. FOWLER, 1935. Permeability of *Amoeba proteus* to water. *J. Cell. Comp. Physiol.*, **6**: 151–167.

ORMSBEE, R. A., 1942. The normal growth and respiration of *Tetrahymena geleii*. *Biol. Bull.*, **82**: 423–437.

PACE, D. M., AND E. D. LYMAN, 1947. Oxygen consumption and carbon dioxide elimination in *Tetrahymena geleii* Furgason. *Biol. Bull.*, **92**: 210–216.

PÈTERFI, T., AND O. MALECI, 1938. Il volume delle cellule gangliari misurato con un nuovo metodo. *Boll. Soc. Italiana Biol. Sper.*, **13**: 708–709.

PRINGSHEIM, E. G., 1936. Zur Kenntnis saprotropher Algen und Flagellaten. 1. Mitteilung. *Arch. Protist.*, **87**: 43–96.

SCHOLANDER, P. F., C. L. CLAFF AND S. L. SVEINSSON, 1952. Respiratory studies of single cells. I. Methods. *Biol. Bull.*, **102**: 157–177.

SCHOLANDER, P. F., R. HOCK, V. WALTERS AND L. IRVING, 1950. Adaptation to cold in arctic and tropical mammals and birds in relation to body temperature, insulation and basal metabolic rate. *Biol. Bull.*, **99**: 259–271.

ZEUTHEN, E., 1943. A Cartesian diver micro respirometer with a gas volume of 0.1 µl. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **24**: 479–518.

ZEUTHEN, E., 1947. Body size and metabolic rate in the animal kingdom. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **26**: 17–161.

ZEUTHEN, E., 1948. A Cartesian diver balance weighing reduced weights (R.W.) with an accuracy of ± 0.01 γ. *C. R. Travaux Lab. Carlsberg, sér. chim.*, **26**: 243–266.